The Role of Intracellular Complement in the Induction of Ineffective CD4+ T-Cell Mediated Immune Responses in Myelodysplastic Syndrome (MDS)

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The Role of Intracellular Complement in the Induction of Ineffective $CD4^+$ T-Cell Mediated Immune Responses in Myelodysplastic Syndrome (MDS)

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Thesis Presented for the Degree of Doctor of Philosophy

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Declaration

I hereby declare that I alone composed this thesis and that the work is my own, except where stated otherwise.

Samia T AL-Shouli

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Aknowledgment

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Abbreviation

α	Alpha
AKT	Protein Kinase B (PKB)
AML	Acute Myeloid Leukemia
AP	Antigen presenting
APCs	Antigen presenting cells
ASXL1	Additional Sex Combs Like 1
β	Beta
β-Actin	Beta-actin
Bcl-2	B-cell lymphoma 2
BM	Bone marrow
BSA	Bovine serum albumin
C3a, C5a	Complement 3a, 5a
C3aR/C5aR	Complement receptors
C5L2	C5a anaphylatoxin chemotactic receptor
cAMP	Cyclic adenosine aminophosphate
CASP	Cysteine-aspartic acid protease
CCUS	Clonal Cytopenia of Undetermined Significance
CD3	Cluster of Differentiation 3
CD28	Cluster of Differentiation 28
CD40L	Cluster of Differentiation40 ligands
CD45RA	Cluster of Differentiation 45RA
CD45RO	Cluster of Differentiation 45RO
CD46	Cluster of Differentiation 46
CHIP	Clonal Haematopoiesis of Indeterminate Potential
CMML	Chronic myelomonocytic leukaemia
CMV	Cytomegalovirus
CO2	Carbon dioxide
CRs	Complement receptors
CTLs	Cytotoxic T lymphocytes

CYT-1 or CYT-2	Cytochrome -1 or 2
δ	Delta
DAF	Decay acceleration factor
DAMPs	Danger-associated molecular patterns
DCs	Dendritic Cells
del5q	Deletion 5q syndrome
DKC	dyskeratosis congenita
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMTs	DNA methyl transferases
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DSM B	Diluted Standard Mix B
ECL	Electrogenerated chemiluminescence
ERK	Extracellular signal-regulated kinase
F (B, D)	Complement Factor B, D
FAS-L	FAS receptor with its specific ligand
FAB	French American British
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FDR	False discovery rate
FGF	Fibroblast growth factor
Foxo1	Forkhead box protein O1
FOXP3	Fork head box P3
FSC	Forward scatter dot plots
Gamma	γ
g/dL	gram/decilitre
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GPCR	G protein-coupled receptors
HBSS	Hanks' balanced Salt Solution
HD	Healthy donors
HHMPW	Hand-Held Magnetic Plate Washer
HR	High-Risk
hr	Hour
HRP	Horseradish peroxidase
HSV	Herpes Simplex Virus
HTA	Human Tissue Authority
IC	Intra-cellular
ICUS	Idiopathic Cytopenias of Undetermined Significance
IFN-α	Interferon-alpha
IFN- γ	Interferon-gamma
IgG-HRP	Immunoglobuline G- horseradish peroxidase
IkBzeta	NFKB Inhibitor Zeta
IL	Interleukin
(i) NKT	Invariant Natural killer T-cells
iNOS	Inducible isoform nitric oxide synthases
IPA	Ingenuity Pathway Analysis
IPSS	International Prognostic Scoring System
IPSS-R	International Prognostic Scoring System revision
iTreg	Induced regulatory T-cells
JAK2	Janus Kinase 2
KDa	Kilo-dalton
LGL	Large granular lymphocytic (LGL) leukaemia
Lin	Lineage
LIR1	Leukocyte Immunoglobulin- like receptor 1
LPS	Lipopolysaccharides
LR	Low-risk
mABs	Monoclonal antibodies
MCF7	Michigan Cancer Foundation-7

MDS	Myelodysplastic Syndrome
MDS-EB	Myelodysplastic syndrome with excess blasts
MDS-MLD	Myelodysplastic syndrome with multilineage dysplasia
MDS-RS	Myelodysplastic syndrome with ring sideroblasts
MDS-RS-MLD	MDS-RS with multilineage dysplasia
MDS-RS-SLD	MDS-RS with single lineage dysplasia
MDS-SLD	Myelodysplastic Syndrome with single lineage dysplasia
MDS-U	Myelodysplastic syndrome-unclassified
MFI	Mean fluorescence intensity
mg/m^2	milligram per square meter
µg/ml	micro-gram/milliliter
MHC	Major histocompatibility complex
miR	microRNA
µL	microliter
ml	Milliliter
mTOR	Mammalian target of rapamycin
mTORC	mTOR Complex
MW	Molecular weight
NaCl	Sodium Chloride
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural Killer Cells
NKG2D	Natural-Killer group 2 member D
NKT	Natural killer T cells
NLRs	Nucleotide oligomerization domain (NOD)-like receptors
NLRP3	NLR family pyrin domain-containing 3
nM	Nano-molar
nTreg	Natural T-regulatory cells
OS	Overall Survival
PAMP	Pathogen-associated molecular patterns
PB	Peripheral blood

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PI3Kγ	Phosphoinositide 3- OH kinase-γ kinase
PKA	Protein Kinase A
MHC I or II	MHC-I complexes
PNH	Paroxysmal nocturnal haemoglobinuria
POU5F1	POU Class 5 Homeobox 1
PRRs	Pattern-recognition receptors
p-S6	Phosphorylation of S6
PYCARD	Apoptosis-associated speck-like protein containing a CARD
RA	Refractory anaemia
RAEB	Refractory anaemia with excess blasts
RAEB-T	Refractory anaemia with excess blasts in transformation
RANTES	Regulated on activation, normal T-cell expressed secreted
RARS	Refractory anaemia with ringed sideroblasts
RARS-T	Refractory anaemia with thrombocytosis
RCMD	Refractory cytopenia with multilineage dysplasia
RIN	RNA intergrity number
RMA	Robust Multi-array average
RNA	Ribonucleic acid
RNAi	RNA-mediated interference
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute
RT	Room temperature
SAPE	Streptavidin-Phycoerythrin
SCR	Short consensuses repeat
SCRs	Short consensus repeats’
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SF3B1	Splicing factor 3B subunit 1
Smad	Suppressor of Mothers Against Decapentaplegic

SSC	Side scatter dot plots
STAT	Signal Transducer And Activator Of Transcription
Stds	Standards
Tconv	Conventional T-cells
TCR	T-cell receptor
TET2	Ten-Eleven Translocation-2
TGF-β	Transforming growth factor beta
TGFbRII	Transforming growth factor beta receptor II
Th1, Th2, Th9, Th17	T-helper cells
TLR4	Toll-like receptor 4
tMDS	Therapy-related MDS
TNF-alpha	Tumor necrosis factor-alpha
TP53	Tumour protein 53
Tr1	T regulatory type 1 cells
Tregs	Regulatory T-cells
TREM-1	Triggering receptor expressed on myeloid cells 1
UAB	Universal Assay Buffer
USA	United State Of America
V	Variable
VEGF	Vascular endothelial growth factor
WB	Western blot
WHO	World Health Organisation
WR	Working reagent
WDR3	WD repeat-containing protein 3
7TM	Seven- transmembrane domain

Abstract

Myelodysplastic syndrome (MDS) is a relatively uncommon clonal haematological disease that is characterised by peripheral blood cytopenias despite a normal or hypercellular bone marrow. The disease transforms to acute myeloid leukaemia in up to 40% of cases. Several studies have established that the T-cell mediated immune dysregulation is an important feature of MDS. Low risk MDS is associated with a pro-inflammatory environment and an increase in IL-17 producing T-cells as well as an increase in the serum IL-17, IL-12, RANTES and IFN-γ. In contrast, IL-10 (inhibitory factor) and IL-2R (soluble receptor) are increased in high risk MDS. A high number of IL-17 producing $CD4^+$ T-cells are found in low-risk MDS compared to the high-risk disease where expansion of T regulatory cells (Tregs) is the main feature. However, the mechanism for this switch in the immune signature is not fully understood. Immune cell derived complement activation fragments are recently identified as key players in driving and modulating adaptive immunity, C3a and C3b. C3 fragments C3a and C5a are particularly important in induction of IFN-γ secretion through autocrine engagement of the C3a receptor and CD46 (C3b receptor). The activation pathway following CD46 activation varies between Tconv and Tregs and the balance between these pathways is crucial for the Treg/Tconv equilibrium. The objective of this project was to elucidate the role of intracellular complement components (C3a and C5a), regulators (CD46) and their receptors (C3aR, C5aR1 and C5aR2) in the expansion of T regulatory cells, which is one of the main factors in the progression of high-risk MDS patients towards AML. In conclusion, this work shows for the first time the potential role of complement in $CD4^+$ T-cells polarisation in MDS patients and provides data on three major pathways that may be implicated in the lack of complement in T-cells of MDS patients. Future work to explore these further may help to identify potential molecules involved which can serve as potential therapeutic targets in MDS in the future.

Chapter 1: Introduction

I. Myelodysplastic Syndrome (MDS)

1.1 Myelodysplastic syndrome (MDS)

Bone marrow failure syndrome consists of a number of overlapping diseases that can either be acquired or inherited. These conditions affect the haematopoietic stem cells and are characterized by variable peripheral blood cytopenias, dysplasia involving either one or multiple cell lines, ineffective haematopoiesis, and often an increased risk of transformation to acute myeloid leukaemia ((Bennett et al., 1982, Vardiman, 2003) (Zeidan et al., 2017)). However, lymphoid lineages are often spared. While Diamond-Blackfan anaemia, Fanconi anaemia, telomeropathies such as dyskeratosis congenita (DKC) and other genetic disorders are inherited bone marrow failure syndromes, idiopathic aplastic anaemia is acquired and is the most frequent cause of acquired bone marrow failure (Kai et al., 2016). Other conditions with presentations similar to acquired bone marrow failure include large granular lymphocytic (LGL) leukaemia, paroxysmal nocturnal haemoglobinuria (PNH), and myelodysplastic syndrome (MDS) (DeZern and Sekeres, 2014).

MDS is a clonal haematological disease that is characterised by peripheral blood cytopenias despite a normal or hypercellular bone marrow. MDS can transform to acute myeloid leukaemia in up to 40% of cases ((Vardiman, 2003);(Yoshizato and Makishima, 2016). The prevalence of MDS is approximately 2 to 12 per 100,000 annually. Prevalence increases with age to 30-50-cases amongst subjects aged 70 years or above (Rollison et al., 2008).

Given the aging populations particularly in the developed countries, MDS is likely to pose a major health challenge. Several lines of evidence have established that MDS is a stem cell disease. Driver mutations in the stem cells lead to a growth advantage for the clone with a marked reduction in the differentiation potential, leading to hypercellular bone marrow,

increased apoptosis of bone marrow haemopoeitic cells and a consequent peripheral blood cytopenias (Corey et al., 2007, Gattermann et al., 2008, Mian et al., 2015).

A specific subset of MDS, referred to as therapy-related MDS (tMDS), can develop following previous chemotherapy and/or radiotherapy exposure, with peak incidence often between 2-4 years after the first exposure (Raposa and Varkonyi, 1987). About 30% of MDS refers to primary MDS. tMDS subset is a more aggressive form of the disease with rapid progression to often chemo-resistant AML and poor survival (Rau et al., 2012). A common characteristic of both MDS and AML is ineffective haematopoiesis, often linked to genetic lesions (Nimer, 2006). A broad range of mutations affecting several genes contributes to the heterogeneity of the disease making its pathophysiology relatively complex (Galili et al., 2009). In addition, abnormalities in innate and adaptive immunity are important in defective immune surveillance, immune tolerance and subversion. Collectively these abnormalities allow the unrestricted proliferation of the dysplastic clones (Voulgarelis et al., 2004). To this end it is interesting to note that several studies have documented the association of MDS with several autoimmune diseases, such as seronegative arthritis (Castro et al., 1991, Chandran et al., 1996, Saif et al., 2002), inflammatory bowel disease, scleroderma, chondritis and sweets syndrome (Saif et al., 2002, Hu et al., 2014), among many others (Saif et al., 2002, Billstrom et al., 1995). Poor survival is often reported in MDS patients in association with autoimmunity (Saif et al., 2002).

1.2 Historical Context of MDS

The story of MDS goes back to 1898 (M.Lowenthal, 2011, Layton and Mufti, 1986). MDS was first acknowledged in the early 1900s, but the usage of the term MDS became prominent in 1976 when the French American British (FAB) morphology group used the term to distinguish this group of relatively chronic haematological diseases from acute myeloid leukaemia **(Table 1-1)** (Bennett et al., 1976). In 1900, Leube described a patient with severe megaloblastic anaemia, followed by the development of overt leukaemia. This was followed by similar reports of patients characterized by cytopenia, dysmaturation of marrow precursors, increase in

marrow blasts, and significant risk of development to acute myeloid leukaemia (Hellstrom-Lindberg, 2008). Ernst Meumann (1834-1918) was attributed with the important discovery that bone marrow (BM) is the site of blood formation. During the same era, the first description of the various blood cells and their morphology was made by a German scientist/physician Paul Ehrlich (1879) (Lowenthal, 2011). Soon after, in 1923, Giovanni Di Guglielmo, a physician in Naples, Italy, reported a number of bone marrow disorders attributed to bizarrely-shaped red blood cells that resulted in low blood count, in particular anaemia, which in some cases proved fatal in the long term (Steensma, 2011). For a long time, all the bone marrow disorders associated with anaemia and abnormal-appearing cells were known *en masse* as "Di Guglielmo Syndrome" by doctors, a term that is still sometimes used for a specific type of leukaemia, erythroleukaemia (AML M6). In 1938, 100 patients with refractory anaemia were reported by C.P. Rhoads and W. Halsey Barker, who were based in New York City (Steensma, 2011). Following that, in 1949, a London pathologist, J.L. Hamilton-Paterson, reported that some cases of refractory anaemia had a propensity to develop into leukaemia; he called this condition 'pre-leukemic anaemia'. This finding was backed by several French investigators, who also came to similar conclusions that refractory anaemia could progress into leukaemia. In 1953, a prominent paper by three physicians from the University of Chicago's Medical School described twelve patients with 'pre-leukemic acute human leukaemia' in the Journal of the American Medical Association. Thus, in the 1960s and 70s, what we now know as MDS was widely known as 'preleukaemia' (Steensma, 2011). Bjorkman was a pioneer in the study of sideroblastic anaemia in 1956; he described four cases of refractory anaemia with 'great amounts of free iron in the normoblasts' and the presence of perinuclear mitochondrial iron, giving the appearance of rings. By 1976, however, Dr John Bennett from Rochester, New York, and six other pathologists from France, the United States and Great Britain - the so-called FAB co-operative group had noticed that most patients with preleukaemia never actually went on to develop leukaemia (Hellstrom-Lindberg, 2008). Then, in 1974, Van Den Berghe described the 5q- syndrome as the first entity within the group of refractory cytopenias with an interstitial deletion of 5q (Hellstrom-Lindberg, 2008). This entity is more common in females,

who have macrocytic anaemia, with a normal or elevated platelet count, typical megakaryocyte morphology and a low incidence of AML transformation. The first classification of leukaemias, including MDS, was made by FAB in 1976 and revised in 1982 to expand its applicability to MDS **(**Lowenthal, 2011, Bennett et al., 1985).

Table 1-1: The French American British (FAB) Classification of MDS (1982)

French-American-British (FAB) cooperative group criteria for myelodysplastic syndrome subtypes					
Type	Bone marrow blasts, percent	Peripheral blood blasts, percent	Auer rods	Monocytes >1000/microL	Ringed sideroblasts, >15 percent of nucleated erythroid cells
RA	< 5	≤ 1	NO	NO	No
RARS	< 5	≤ 1	NO	NO	Yes
RAEB	5 t0 20	< 5	NO	NO	±
CMML	≤20	< 5	NO	Yes	±
RAEB-T*	21 to 30	≥5	±	±	±

RA: refractory anaemia; RARS: refractory anaemia with ringed sideroblasts; RAEB: refractory anaemia with excess blasts; CMML: chronic myelomonocytic leukaemia; RAEB-T: refractory anaemia with excess blasts in transformation (Vardiman, 2012).

In 1976, the term 'dysmyelopoietic syndrome' was proposed by the FAB group as an alternative to preleukaemia. A few years later this was revised to 'myelodysplastic syndrome' and classified in 1982, again by the FAB group (Steensma, 2011). This classification included the following subtypes: refractory anaemia (RA), refractory anaemia with ring sideroblasts (RARS), refractory anaemia with excess blasts (RAEB), refractory anaemia with excess blasts in transformation (RAEBT), and chronic myelomonocytic leukaemia (CMML). In the recent World Health Organization (WHO) classification, CMML and RAEB-T were removed from the MDS classification and RAEB was split into two groups with medullary blast counts below and above 10% (Gupta et al., 2010).

During the 1990s it became evident that additional clinical variables, and in particular chromosome analysis, could contribute significantly to clinical outcomes and that survival and risk of leukemic transformation was substantially worse in patients with adverse cytogenetics (Hellstrom-Lindberg, 2008).

In 1997, Greenberg *et al*. introduced the International Prognostic Scoring System (IPSS), the database which was based on a wide range of information gathered from patient samples from Europe, the United States, and Japan (Greenberg et al., 1997). The first WHO classification was developed in 2000 and revised in 2008 (Vardiman et al., 2009). The current classification of MDS used by doctors globally is the WHO's 2008 version, which was updated in 2016.

1.3 MDS classification

In recent years the morphological assessment of the peripheral blood and bone marrow, has been supplemented by the cytogenetic, molecular and immunophenotypic information. This is reflected in the 2008 WHO classification of MDS that uses a combination of clinical, genetic, immunophenotypic and morphological features from peripheral blood and bone marrow (Vardiman et al., 2009) and identifies six subtypes. Refractory cytopenia (RA), refractory anaemia with ringed sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), refractory anaemia with excess blasts (RAEB1 and RAEB2), myelodysplastic syndrome unclassifiable and myelodysplastic syndrome with isolated del5q (5q syndrome) (Vardiman et al., 2009). MDS with isolated del (5q) show features of '5q-syndrome', characterised by an interstitial deletion of the long arm of chromosome 5, macrocytic anaemia, low probability of leukaemic transformation and normal/elevated platelet count (Vardiman et al., 2009). The updated 2016 WHO classification **(Table 1-2)** eliminates explanations of the cytopenic lineage (e.g., refractory anaemia) in favour of an account of the dysplastic lineages (MDS with single lineage dysplasia). Erythroid-predominant cases no longer bid for separate blast enumeration of the non-erythroid cells, a difference that will mark the reclassification of most acute erythroid leukaemia and erythroid/myeloid types as MDS.

Table 1-2: The (2016) World Health Organization (WHO) classification of MDS: Peripheral Blood (PB) and Bone Marrow (BM) findings and cytogenetics of MDS (Arber et al., 2016).

Name	Dysplastic lineages	Cytopenias *	Ring Sideroblasts as % of marrow erythroid elements	BM and PB blasts	Cytogenetic by conventional karyotype analysis
MDS with single lineage dysplasia (MDS-SLD)	1	1 or 2	<15%/<5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with multilineage dysplasia (MDS-MLD)	2 or 3	1-3	<15%/<5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with ring sideroblasts (MDS-RS)					
MDS-RS with single lineage dysplasia (MDS-RS-SLD)	1	1 or 2	>_15%/_>5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS-RS with multilineage dysplasia (MDS-RS-MLD)	2 or 3	1 - 3	>_15%/_>5%†	BM <5%, PB <1%, no Auer rods	Any, unless fulfills all criteria for MDS with isolated del(5q)
MDS with isolated del(5q)	1 - 3	1 - 2	None or any	BM <5%, PB <1%, no Auer rods	del(5q) alone or with 1 additional abnormality except -7 or del (7q)
MDS with excess blasts (MDS-EB)					
MDS-EB-1	0-3	1-3	None or any	BM 5%-9% or PB 2%-4%, no Auer rods	Any
MDS-EB-2	0-3	1-3	None or any	BM 10%-19% or PB 5%-19% or Auer rods	Any
MDS, unclassifiable (MDS-U)					
With 1% blood blasts	1-3	1-3	None or any	BM <5%, PB =1%‡, no Auer rods	Any
With single lineage dysplasia and pancytopenia	1	3	None or any	BM <5%, PB <1%, no Auer rods	Any
Based on defining cytogenetic abnormality	0	1-3	<15%§	BM <5%, PB <1%, no Auer rods	MDS-defining abnormality
Refractory cytopenia of childhood	1-3	1-3	None	BM <5%, PB <2%,	Any

*Cytopenias defined as: haemoglobin, 10 g/dL; platelet count, 100 x 10^9/L; and absolute neutrophil count, 1.8 x 10^9/L. On rare occasions MDS may exhibit mild anaemia or thrombocytopenia above these levels. PB monocytes must be < 1 x 10^9/L. †If SF3B1 mutation is present. ‡1% PB blasts must be recorded on at least 2 separate occurrences. §Cases with >_ 15% ring sideroblasts have significant erythroid dysplasia, and are classed as MDS-RS-SLD (Arber et al., 2016).

SF3B1 mutation, if present, enables a diagnosis of MDS with ring sideroblasts when even fewer than 5% ring sideroblasts are displayed. A diagnosis of MDS with isolated del (5q) may possibly be made if one additional cytogenetic abnormality is present, with the exclusion of monosomy 7 or del (7q). For the time being, the detection of other somatic gene mutations are excluded as a diagnostic criterion for MDS, because of the challenges posed by age related Clonal Haematopoiesis of Indeterminate Potential (CHIP) (Arber et al., 2016), Idiopathic Cytopenias of Undetermined Significance (ICUS) and Clonal Cytopenia of Undetermined Significance (CCUS). CHIP is characterised by the acquisition of somatic mutations that drive clonal expansion in the absence of cytopenias and dysplastic haematopoiesis. The most frequently mutated genes associated with CHIP are DNMT3A, TET2, and ASXL1 (Abdel-Wahab and Levine, 2013). Most individuals who acquire clonal haematopoiesis in later ages never progress to developing MDS (Steensma et al., 2015). The absence of cytopenias and dysplastic haematopoiesis differentiates CHIP from MDS. ICUS patients on the other hand are characterised by persistent blood cytopenias of unknown cause, and display unremarkable marrow morphology that lack a known MDS-associated somatic mutation or karyotypic abnormality (Valent et al., 2012). Some individuals with ICUS have been shown to have progressed and developed MDS or AML (Hanson, 2009). CCUS patients are characterised by unexplained cytopenias and a clonal mutation, who however do not meet WHO defined criteria for MDS or other heamatologic neoplasm (Malcovati and Cazzola, 2015, Steensma et al., 2015). Prognostic systems, such as the IPSS (Greenberg et al., 1997) (**Table 1-3**) and its revision (IPSS-R) **(Table 1-4)** have been developed and validated for MDS patients (van Spronsen et al., 2017, Della Porta et al., 2015, Greenberg et al., 2012). Three prognostic indicators are used by the IPSS to predict the course of the patient's disease. There are three variables; bone marrow blasts, karyotype and cytopenia which are utilised to predict survival and AML evolution (Greenberg et al., 1997, Lim et al., 2007, Gupta et al., 2010).

The patient's IPSS risk category is determined by adding up the IPSS scores within the three disease factors, which matches the patient to one of the following four IPSS risk categories:

low, intermediate-1, intermediate-2 or high. At times the low and intermediate-1 categories are combined into a lower risk group, while the intermediate-2 and high categories are together denoted as higher risk group.

The modified IPSS, known as the IPSS-R, covers the same disease factors as the IPSS but in greater detail particularly in relation to cytogenetic abnormalities **(Table 1-4).** The IPSS-R was established using data sets from an initial group of 7,012 patients with primary MDS; this system enabled MDS patients to be classified into five groups (from very low to very high) showing varied estimated overall survival and progression to AML (Greenberg et al., 2012). In addition to cytogenetics, values for haemoglobin and platelet counts are given different score depending on whether the level is <8 or ≥ 10 in the case of former and <50 or ≥ 100 in the case of later. A patient's IPSS-R risk category is established by tallying the individual IPSS-R scores for the defined values within the five disease subgroups. Five IPSS-R risk classes are defined as very low, low, intermediate, high and very high.

Table 1-3: International prognostic scoring system (IPSS) classification of MDS

International Prognostic Scoring System (IPSS)	Points				
Prognostic variable	0	0.5	1	1.5	2
Bone Marrow blasts (%)*	< 5%	5%-10%	-	11%-19%	20% - 30%
Cytopenias**	0 - 1	2-3	-	-	-
Cytogenetic Category (Karyotype)***	Good	Intermediate	Poor		

International Prognostic Scoring System (IPSS)	Median Survival (years)			
Risk Groups	Score	<_ 60	> 60	All Patients
Low	0	11.8	4.8	5.7
Intermediate I	0.5 - 1	5.2	2.7	3.5
Intermediate II	1.5 - 2	1.8	1.1	1.2
High	>_ 2.5	0.3	0.5	0.4

* Bone marrow blasts of 20-30% may imply MDS (according to the French-American-British (FAB) classification) or AML (according to WHO). ** Cytopenia defined as neutrophils $<1.8x10^9$ /L, platelets $<100x10^9$ /L, haemoglobin <10g/dl. *** Cytogenetics (Karyotype): Good = normal, -Y alone, del (5q) alone or del (20q) alone; Poor = complex (>_3 abnormalities) or chromosome 7 abnormalities (Greenberg et al., 1997, Gupta et al., 2010).

Table 1-4: Revised International prognostic scoring system (IPSS-R) classification of MDS

IPSS-R Cytogenetic risk groups	
Cytogenetic prognostic subgroups	**Cytogenetic abnormalities**
Very good	-Y, del(11q)
Good	Normal, del(5q), del(12p), del(20q), double including del(5q)
Intermediate	del(7q), +8, +19, i (17q), any other single or double independent clones
Poor	-7, inv(3)/t(3q)/del(3q), double including -7/del(7q), Complex: 3 abnormalities
Very poor	Complex: > 3 abnormalities

IPSS-R Prognostic Score Values							
Prognostic variable	**0**	**0.5**	**1**	**1.5**	**2**	**3**	**4**
Cytogenetic	Very good		Good		Intermediate	Poor	Very poor
BM blast, %	< 2		> 2%- < 5%		5%-10%	>10%	
Hemoglobin	> 10		8 < 10	< 8			
Platelets	> 100	50 < 100	< 50				
ANC	>0.8	<0.8					

IPSS-R Prognostic Risk Categories/ Scores	
Risk Category	**Risk Score**
Very Low	< = 1.5
Low	> 1.5-3
Intermediate	> 3-4.5
High	> 4.5-6
Very High	> 6

Distribution of IPSS-R Categories and their Outcomes						
	Number of Patients	**Very Low**	**Low**	**Intermediate**	**High**	**Very High**
Patients (%)	7012	19%	38%	20%	13%	10%
Survival***		8.8	5.3	3	1.6	0.8
AML/25%***		NR	10.8	3.2	1.4	0.7

***Medians, years; AML/25% (Median time to 25% AML evolution (Greenberg et al., 2012, Pardanani and Tefferi, 2012).

1.4 Implication of Cytogenetic abnormalities in MDS

Cytogenetic abnormalities provide important information with respect to the diagnosis, prognosis and appropriate treatment options in patients with MDS. Several chromosomal deletions are associated with the development of MDS and its associated complications. MDS is characterized by frequent cytogenetic aberrations such as deletions on the long arms on chromosomes 5, 7 and 20, as well as more complex karyotypes such as trisomy 8 and other chromosomal lesions such as del Y, 20q-, 3q26 (Gerstung et al., 2015).

Chromosome 5q deletion (5q- syndrome) was first reported over four decades ago (Van den Berghe et al., 1974) and is a distinct subtype of MDS characterised by the presence of an isolated interstitial deletion of chromosome 5q and <5% blasts in the bone marrow (Boultwood et al., 1994, Giagounidis et al., 2004). 5q- syndrome is also regarded as a disease of haematopoietic stem cell (Gordillo et al., 2008). In patients with MDS, the 5q- syndrome is the most common cytogenetic abnormality, with an incidence of between 10-15% of MDS patients carrying this mutation either as the sole karyotypic abnormality or in combination with other chromosomal abnormalities (Sole et al., 2000, Heim and Mitelman, 1986).

Chromosome 7 and 7q deletions are a distinct subtype of MDS characterised by the deletion of part of the long arm of chromosome 7 (7q), total loss of chromosome 7 (monosomy 7), or translocations involving chromosome 7, and can be present either as a single anomaly or in combination with other anomalies such as -5/del(5q) and trisomy 8 (Cordoba et al., 2012). Either in isolation or as a part of a complex karyotype, about 10% of patients harbour abnormality of chromosome 7 and this incidence increases to 50% in tMDS patients with alkylating agent's treatment history (Christiansen et al., 2004). In terms of prognosis, MDS patients with 7q deletion have been shown to be associated with fewer blasts in bone marrow, and significantly higher survival superiority (i.e. better prognosis) than in those with isolated monosomy 7 (Cordoba et al., 2012). Moreover, other evidence shows that the outcome is better

when -7 or del (7q) occurs in MDS patients with a simple karyotype, compared with those with a complex karyotype (Woo et al., 2009).

Trisomy 8 is a fatal condition characterised by the presence of three copies of chromosome 8 in every cell of an individual's body (Gilbert-Barness E, 1991). The condition is present as a sole abnormality in approximately 8% of the patients and is the only recurrent observed chromosomal amplification. It is observed as an intermediate cytogenetic abnormality. Compared with patients with normal karyotype, individuals with Trisomy 8 have a life span less than half the median expected survival. The clone size influences the prognostic factor in MDS, and in Trisomy 8 patients with a clone size (aberrant metaphases) of 100% have been shown to have a shorter survival (Mallo et al., 2011).

Chromosomal lesions such as del Y, 20q-, 3q26 have been reported in MDS (Nardi and Hasserjian, 2016). Both –Y and 20q are regarded as being in the same favourable cytogenetic risk group as patients with a normal karyotype (Yun et al., 2011). There is no correlation between the loss of chromosome Y with disease pathogenesis while interstitial loss of 20q appears to be pathogenic not only in MDS but also myeloproliferative disorders and AML (Wiktor et al., 2000).

The most frequent cytogenetic abnormalities along with their frequency of incidence and prognostic impact are detailed in **(Table 1-5)** below:

Table 1-5: Common cytogenetic abnormalities and their frequency of incidence in MDS and their impact on prognosis (Haase et al., 2007, Bejar, 2014).

Chromosomal abnormalities	Frequency (%)	Prognosis
Trisomy 8	5 to 8	Good
Trisomy 21	2.7	Good
del 5q	15	Good
del 20q	2 to 5	Good
del Y	0.8	Good
-7/del 7q	5 to 10	Intermediate to poor
del 17p	10	Poor
Complex (≥3 abnormalities)	13.4	Poor

1.5 MDS Therapeutic Implications

Owing to the heterogeneous nature of MDS, treatments are tailored to the predicted prognosis for each patient; hence the accurate prediction of the prognosis is essential component in the overall patient care. MDS are mostly diagnosed in the elderly (median age range 65 to 70 years). The International Prognostic Scoring System (IPSS-R) is a widely used tool to assess the risk of MDS transformation to leukaemia and to guide treatment decisions by physicians. Both karyotypic abnormalities and patient's clinical features are considered in determining the risk of an individual. IPSS-R takes into account the patient's age and associated co-morbidities, patient's expectations and personal goals. In younger age patients with high risk of disease, stem cell transplantation and chemotherapy are usually considered. MDS patients need both supportive care/therapy as well as curative treatment options. Care of MDS patients involve both supportive care and curative treatment options.

Haemoglobin levels in MDS patients are generally maintained between 8 and 10 g/dL. Patient with a low or intermediate-1 risk and having a haemoglobin level greater than 10 g/dL and platelet counts of 50,000/μL to 100,000/μL do not usually need blood transfusion; these patients are often classified 'watch and wait' with regular observation in monitoring them (Ria et al., 2009). Supportive therapy for severe MDS patients may include blood transfusions to replenish red cells and platelets based on clinical evaluation of anaemia-related symptoms and any associated secondary infections (Ria et al., 2009), reducing cytopenia, and improving the quality of life through the administration of immunosuppressive drugs, iron chelation therapy, treatment of infectious or heamorrhagic complications, and psychological support to patients and relatives (Sanchez, 2011). The potential danger of iron overload in the elderly due to transfusion may prompt the requirement of iron chelating agents, with the administration of oral deferasirox (Exjade®: 20 to 40 mg/kg per day) in transfusion-dependent MDS patients seen to induce a significant reduction of the mean serum ferritin after 12 months of therapy (List et al., 2012). Erythropoiesis-stimulating agents such as recombinant human

erythropoietins used as a monotherapy in doses ranging from 30,000 to 60,000 units/week induced erythroid responses in about 15% of MDS patients (Hellstrom-Lindberg, 1995). Furthermore, the pancytopenia observed in most MDS patients warrants the prophylactic use of granulocyte colony-stimulating factor in patients with severe neutropenia and recurrent infections (Ria et al., 2009). Severe thrombocytopenia with chronic bleeding is common in MDS and is associated with shortened survival and impaired quality of life (Ria et al., 2009). Platelet transfusions may therefore be necessary to provide short-term relief for bleeding tendencies. The Food and Drug Administration (FDA) approved drug AMG 531 is effective for the treatment of immune thrombocytopenia MDS patients (Tiu and Sekeres, 2008). DNA methyltransferase (DNMT) are involved in the post-mitotic modification of DNA methylation, while in cancers and conditions such as MDS the mechanisms controlling methylation can become dysregulated.

Demethylating agents have been shown to be the standard therapy for patients with higher-risk MDS and the currently known agents to improve the natural history of MDS (Garcia-Manero, 2008). Demethylating agents are a group of chemotherapeutic agents that are able to induce transient DNA hypomethylation. The FDA has in recent times approved three hypomethylating agents for the treatment of MDS, these are 5- azacitidine, 5-aza-2′-deoxycitidine (decitabine), and the immunomodulatory drug Lenalidomide (Diesch et al., 2016, Silverman et al., 2002). These agents have significant activity in patients with higher risk MDS and are widely used in the USA and other European countries. In 5q- type of MDS, lenalidomide is often used as the first treatment choice followed by, treatment with azacitidine or decitabine option lenalidomide fails. Contrary, treatment with azacitidine or decitabine is often the first choice for patients with the non 5q- type MDS.

Lenalidomide is an immunomodulatory drug that is a synthetic compound derived by modifying the chemical structure of thalidomide; it is a 4-amino-glutamyl analogue of thalidomide but lacks the neurologic side effects of sedation and neuropathy associated with thalidomide. Lenalidomide has different mechanisms in different haematological disorders.

Although the exact mechanism of action of lenalidomide on del 5q- MDS patients is unknown it is widely known to modulate different components of the immune system by altering cytokine production, regulating T-cell co-stimulation and augmenting the NK cell cytotoxicity (Kotla et al., 2009).

In high-risk MDS (intermediate-2 and high IPSS), it is important to modify the natural history of the disease by eliminating the malignant clone to prevent the early progression to AML and the associated short median overall survival in these groups. Bone marrow transplantation is usually considered the only curative option for these patients, especially younger patients with MDS (recommended treatment for nearly all children), and is usually performed as soon as possible, although it requires that a matched donor to found (Sierra et al., 2002, Cutler et al., 2004). Such procedures have associated morbidity and mortality, including the possible mutations of genes in allogenic bone marrow transplant patient. For example, a recent study found at least 1 mutation in 1196 of 1514 patients (79%) who had undergone bone marrow transplantation, with a median of 2 driver mutations per patient (range, 0 to 15) (Lindsley et al., 2017). The authors found that mutations such as *TP53* and *DNMT3A* that are associated with higher-risk MDS according to the IPSS, were more prevalent in patients with MDS who had undergone transplantation than those who did not receive any transplant, whereas mutations that are associated with lower-risk MDS, such as *SF3B1,* were less prevalent. The *TP53* mutation was significantly associated with shorter overall survival compared with patients with no mutations who had prolonged survival (Lindsley et al., 2017).

II. Immune system in MDS

1.1 The clinical evidence of Autoimmunity in MDS

The pathophysiology of MDS remains poorly defined; this is attributable to the heterogeneric nature of the disease. Genetic mutations, and clonal karyotypic abnormalities are a hallmark of this group of diseases. In addition to these, several lines of evidence suggest that defective innate and adaptive immune responses are a common feature of MDS including altered cytokine/chemokine profile which is also implicated in MDS pathogenesis. Several studies have reported strong association between autoimmunity and MDS, with up to 10% of MDS patients reported to have a concomitant autoimmune disorders (Mufti et al., 1985, Mufti and Galton, 1986, Hamblin, 1996). That include such as vasculitis, arthritis, peripheral neuropathy, pulmonary infiltrates, inflammatory bowel disease, connective tissue disorders, glomerulonephritis, thyroid abnormalities, sweets syndrome and autoimmune cytopenias (Castro et al., 1991, Billstrom et al., 1995, Saif et al., 2002). Clinical evidence to support the association of autoimmunity with MDS include the presence of both organ and non-organ specific autoantibodies in over a fifth of MDS patients, the presence of both monoclonal gammopathies and polyclonal hypergammaglobulinaemia in just above a tenth of MDS patients and hypogammaglobulinaemia in half of all MDS cases (Mufti et al., 1986).

1.2 Innate Immune cells in MDS

1.2.1 Natural Killer Cells (NK cells) and MDS

As part of the innate arm of immune system natural killer (NK) cells are important part of the first line of defence against both intracellular pathogens and tumour cells (Grossenbacher et al., 2017, Stabile et al., 2017). NK cells regulate tumour initiation via their ability to recognize and kill malignant cells, and synchronise with the adaptive immune response by releasing cytokines and chemokines (Stabile et al., 2017). A few studies have explored the contribution of NK cells in MDS, with some reports of normal levels but impaired function of

NK cells (Epling-Burnette et al., 2007a, Kiladjian et al., 2006, Chamuleau et al., 2009). These impaired function of NK cells in high-risk MDS may facilitate disease progression. In one of the above studies, the authors showed that reduced NK cells function in MDS patients was significantly associated with higher International Prognostic Score, abnormal karyotype, the presence of excess blasts, and age-adjusted bone marrow hypercellularity. MDS patients with a display of the activating receptor NKp30, and NKG2D down-regulation closely correlated with impaired NK function (Epling-Burnette et al., 2007a).

NK cells have been found to have high expressions of Leukocyte Immunoglobulin-like receptor 1 (LIR1) (Jain et al., 2017), (an inhibitory molecule) which induces leukaemia-killing impairment (Godal et al., 2010). NK cells are functionally impaired in high-risk MDS (Carlsten et al., 2010, Jain et al., 2017). However, in low and intermediate risk MDS, NK cell expression of granzyme B is high with non-major histocompatibility complex (MHC) directed killing of autologous haematopoietic precursor cells being increased (Chamuleau et al., 2009) **(Table 1-6)**.

Table 1-6: The role of immune responses in MDS

Immune Cells	Low-Risk MDS	High-Risk MDS	Role in MDS
Cytotoxic CD8+ T cells	⇧	⇩	* Mediate bone marrow (BM) precursor cells cytotoxicity in low-risk MDS.
Th17 cells	⇧	⇩	* Mediate bone marrow (BM) precursor cells cytotoxicity in low-risk MDS.
NK Cells	⇧	⇩	* Mediate bone marrow (BM) precursor cells cytotoxicity in low-risk MDS.
CD4+FOXP3+ (Tregs)	⇩	⇧	* Low frequencies of Tregs in low-risk MDS enhance immune cytotoxicity by T-cells. * High number of Tregs in high risk suppresses the immune responses.
NK T Cells	⇧	⇩	* Contradictory role in MDS pathology.
Dendritic Cells (DCs)	⇩	⇩	• Low myeloid and lymphoid circulating precursors DCs frequency in both low-risk and high risk MDS.
Macrophages	⇧	⇩	* IFN- γ producing cells. * In low-risk MDS, macrophages are cytotoxic for bone marrow precursor cells.

The role of cellular innate and adaptive immune responses in MDS depending on whether it is high-risk or low-risk. Increase in number and decrease in frequency (Aggarwal et al., 2011).

1.2.2 iNKT cells and MDS

Although the exact contribution of invariant (i) NKT-cells remains debatable, they are reported to contribute primarily to innate and adaptive immune response modulation via dendritic cell (DC) activation or elimination (Aggarwal et al., 2011). Activated iNKT-cells secrete both inflammatory (e.g. IFN-γ, TNF-α) and anti- inflammatory (e.g. IL-4, IL-13) cytokines, thus providing a potential for preventing autoimmunity as well as promoting host anti-cancer immunity to cancer (Aggarwal et al., 2011, Molling et al., 2008). Although three different studies have shown decreased levels of iNKT-cells in MDS, they were limited by the lack of control age-matched group. Later studies demonstrated that levels of iNKT cell decreased with age (Fujii et al., 2003, Zeng et al., 2002). However, Chan et. al., showed that there was no difference in the levels of iNKT cells between age-matched MDS patients and the healthy control group (Chan et al., 2010) **(Table 1-6)**.

1.2.3 Dendritic cells (DC) and MDS

Dendritic cells (DCs) are professional antigen presenting cells that act as regulators of immunity. DCs are classified as either myeloid and plasmacytoid DC and can be distinguished based on their morphology, surface phenotype and specificity of response to antigenic signals. Both myeloid and plasmacytoid DC stimulate a primary T-cell response to antigen (Knight et al., 2002). Their role in MDS has gained major attention. It has been shown that the absolute numbers of the two DC subsets in the peripheral blood of MDS patients are lower than in healthy controls, and that both populations were derived from the malignant clone (Ma et al., 2004). Monocyte-derived DC have been shown to be clonally involved in MDS patients with refractory anaemia, and refractory anaemia with excess blasts with karyotype abnormalities (Matteo Rigolin et al., 1999). This observation contrast that observed in CMML in which the yield and immunophenotypic maturation of monocyte-derived DC are less dramatically altered (Vuckovic et al., 1999) and in AML where myeloid or lymphoid derived DC numbers can be unaltered, decreased or increased (Mohty et al., 2001) **(Table 1-6)**.

1.2.4 Gamma delta (γδ) T-cells and MDS

γδ T-cells play an important role in the rapid response against both tumour and non-tumour cells and are an important component of lymphoid stress-surveillance response (Hayday, 2009, Band et al., 1990, Kunzmann et al., 2000, Kunzmann and Wilhelm, 2005)). γδ T-cells bearing the Vγ9Vδ2 rearrangement constitute just about a tenth of circulating peripheral blood T-cells, a proportion which even further lowered in MDS patients (Kiladjian et al., 2008). The results were most profound in MDS patients with associated autoimmune disorders. In the above, the authors reported that in a subset of MDS patients the γδ T-cells failed to expand in response to bromohalohydrin pyrophosphate and demonstrated limited proliferative potential in response to IL-2, however, the cytolytic function was preserved in those where expansion was observed (Kiladjian et al., 2008). All these results demonstrate the complexity of the immune dysregulation in MDS.

1.2.5 Inflammasome and MDS

Recent studies have shown that the NLR family pyrin domain-containing 3 (NLRP3) inflammasome plays a major role in MDS by directing clonal expansion and pyroptotic cell death. Independent of genotype, MDS hematopoietic stem and progenitor cells overexpressed inflammasome proteins and manifest activated NLRP3 complexes that direct the activation of caspase-1, IL-1b and IL-18 generation, and pyroptosis (Basiorka et al., 2016).

1.2.6 Monocytes and MDS

Monocytes are professional phagocytic cells of the innate immune system that migrate from the circulation and extravasate through the endothelium, where they differentiate into macrophages. Monocytes generated from T-helper cell activation contribute to immune dysregulation of MDS (Meers et al., 2007). A number of studies have hypothesised that the CD40-CD40 ligand could affect immune-mediated bone marrow (BM) failure in MDS. CD40-stimulation of monocytes in MDS results in high TNF-α secretion in MDS. Furthermore, bone marrow mononuclear cells co-cultured with CD40 blocking antibody increased colony-forming units (Meers et al., 2007). In MDS patients an increased monocyte count, but impaired

ability to induce macrophages, resulting in reduced number of macrophages compared with normal control group has been observed (Han et al., 2016). This reduced macrophage count usually result in increased levels of iNOS secreted by macrophages in MDS (Han et al., 2016).

1.2.7 Macrophages and MDS

Macrophages are innate immune cells derived from monocytes. They are distributed throughout the body tissues, and function to ingest and degrade abnormal cells, debris, and foreign material and orchestrate inflammatory processes. Macrophages engage in the clearance of debris from apoptotic bone marrow haematopoietic cells (Dogusan et al., 2004, Lambert et al., 2016). DAMPs and PAMPs are strong ligands for PRRs (Lambert et al., 2016). The toll-like receptor 4 (TLR4), amongst the PRRs, is over-expressed by macrophages in MDS as well as by stem cells and stromal cells (Lambert et al., 2016, Wei et al., 2013) **(Table 1-6)**.

A high frequency of bone marrow (BM) macrophages is usually observed in MDS (Kitagawa et al., 1993, Sadahira et al., 1999). An increase in the frequency of macrophages in high-risk MDS patients compared to low-risk patients mediates angiogenesis via the production of basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and increased stimulation of pro-inflammatory cytokines, thus possibly explaining the increased cellular death in the BM of LR-MDS patients (Wetzler et al., 1995). Furthermore, elevated levels of soluble angiogenic factors (VGEF) in high-risk MDS patients are reported to contribute to immunosuppression. This suggests that inhibition of activated macrophage function at initial stages of MDS development may improve BM homeostasis in low-risk MDS cases and thereby reduce inflammation associated apoptosis **(Table 1-6)** (Aggarwal et al., 2011).

1.3 The role of the adaptive immune response in MDS: T-cell biology of MDS

1.3.1 Concept of T-cell oligoclonality in MDS and response to immunosuppressive therapy

Oligoclonal is defined as a small group of proteins that migrate close together during electrophoresis producing closely placed bands on the electrophoretogram. In MDS patients increased $CD8^+$ cytotoxic T lymphocytes (CTLs) displaying an effector phenotype are a common feature (Melenhorst et al., 2002, Epling-Burnette et al., 2007b, Kook et al., 2001). A lower $CD8^+$ CTLs count has been observed in high-risk MDS compared to low-risk MDS patients (Epling-Burnette et al., 2007b, Fozza et al., 2009, Sloand et al., 2011, Chamuleau et al., 2009). Although MDS is defined by cytopenias, dysplastic morphology of blood and marrow cells, and clonal haematopoiesis, most individuals who acquire clonal haematopoiesis during aging do not progress to developing MDS (Steensma et al., 2015). Clonal haematopoiesis must be exhibited by a somatic mutation with a variant allele frequency of at least 2% (Steensma et al., 2015). MDS and other haematological disorders such as aplastic anaemia display oligoclonal T-cells (Wlodarski et al., 2006, Risitano et al., 2002). Oligoclonal T-cells are not only a feature of MDS, they can also be seen in other haematological disorders such as aplastic anaemia, T-cell large granulocytic leukaemia and paroxysmal nocturnal haemoglobinuria (PNH) (Wlodarski et al., 2006, Risitano et al., 2002, O'Keefe et al., 2004). In MDS patients, while no correlation has been seen between clonality and disease severity, a correlation between a positive response to immunosuppressive therapy and a loss of clonal T-cells was observed in patients treated with immunosuppressive (Wlodarski et al., 2006). In addition, clonally restricted haematopoiesis is associated with increased risk of subsequent diagnosis of myeloid or lymphoid neoplasia (Steensma et al., 2015).

Several hypotheses have been suggested as the underlying mechanism of autoimmunity in MDS. Some propose that cytomegalovirus (CMV) infection in which prevalence increases with age, may be the cause of the expansion of $CD8^+$ CTLs, while an alternative hypothesis proposes that effector T-cells may be triggered as a result of increased apoptotic cytokines and

aberrant expression of oncogenes or fusion genes in haematopoietic stem cells (Epperson et al., 2001). In addition to immune surveillance, immune responses can promote tumour growth. A number of studies have proposed dysregulation of the immune system in the intricate MDS pathogenesis. Immune dysregulation could potentially promote progression of MDS from lower/early risk to higher/advance risk MDS. Lower-risk MDS is often characterized by autoimmune disease-like features (such as increased T-helper cell type 17 (Th17); increased levels of pro-apoptotic cytokines; low regulatory T-cells (Treg) number; dysfunctional B-cells) while higher risk MDS is marked with low apoptosis, immune invasion, increased Treg and dysfunctional natural killer (NK) cells **(see Table 1-6).** Reports such as that of Pellagatti et al. show that MDS accounts for aberrant immune responses (Pellagatti et al., 2010). This perturbed response results in disease symptoms such as cytopenia often affecting both normal and dysplastic cells (Pellagatti et al., 2010), making treatment quite challenging.

1.4 T-cells dysregulation in MDS

1.4.1 $CD8^+$ cytotoxic T lymphocytes (CTLs)

MDS is characterized by high levels of effector phenotypic $CD8^+$ cytotoxic T lymphocytes (CTLs) (Melenhorst et al., 2002, Epling-Burnette et al., 2007b). MDS and other haematological disorders such as aplastic anaemia display oligoclonal T-cells (Wlodarski et al., 2006, Risitano et al., 2002). Several studies have reported a positive correlation between loss of clonal T-cells in MDS patients and positive response to immunosuppressive therapy (Wlodarski et al., 2006), with some groups reporting high levels of oligoclonal T-cells in MDS in comparison to controls (Kochenderfer et al., 2002). Similarly, a low number of $CD8^+$ CTLs has been observed in high-risk MDS in contrast to low-risk MDS **(Table 1-6)** (Epling-Burnette et al., 2007b, Fozza et al., 2009, Sloand et al., 2011, Chamuleau et al., 2009).

1.4.2 Regulatory T-cells (Tregs)

Tregs cells are characterised by the following phenotypes: $CD4^{+}CD25^{high}FOXP3^{+}$ or $CD4^{+}CD25^{high}CD127^{low}$. Key functions of Tregs include immune tolerance and modulation of immune response during immune surveillance (Wang et al., 2018). These functions are performed via secreting immunosuppressive cytokines such as IL10 and TGF-Beta (Aggarwal et al., 2011). Defective immune activation and a decreased immune surveillance against the tumor cells are characteristic of impaired Tregs (Selma D'Silva, 2019). Moreover, reduced numbers of functional Tregs result in autoimmunity.

Although the exact role of Tregs in MDS progression is not very clear, various reports have linked variation in number of Tregs with disease progression, risk of transformation to AML and overall survival in MDS patients (Mailloux and Young, 2010, Lambert et al., 2016). Tregs involvement in MDS was first shown by Kordasti et al. (Kordasti et al., 2007), in which the authors demonstrated an association between increased $CD4^{+}$ Tregs and disease progression. Various studies have shown an association between low number of Tregs in bone marrow and increased $CD8^{+}$ T-cells and the recruitment of Th17 proinflammatory cells (Alfinito et al., 2010, Kordasti et al., 2009). The number of Tregs however differs based on the stage of the disease. For example, in low-risk MDS, lower numbers of Tregs or impaired Tregs have been reported in association with T-cell cytotoxicity leading to increased apoptosis, whereas, higher number of Tregs are seen in high-risk MDS patients and are associated with impaired antineoplastic immunity and immune suppression (Kotsianidis et al., 2009, Zou et al., 2009).

A study that investigated the effect of 5-azacytidine on Tregs functionality in intermediate/high-risk MDS patients reported that following treatment, lower numbers and Tregs with lower suppressive function were observed in those patients (Costantini et al., 2013). Others have also reported an inverse relationship between levels of Tregs and the degree of dyserythropoiesis (Alfinito et al., 2010), and an inverse ratio between Treg and $CD8^{+}$ frequencies in MDS patients (Hamdi et al., 2009). Low-risk MDS patients have higher number of Th17 cells which is inversely correlated with Tregs numbers. This results in an

increased Th17/Tregs ratio in such patients (Kordasti et al., 2009). Furthermore, an increased number of Th22 (IL22 producing T-cells) in high-risk MDS patients has been reported (Shao et al., 2012) and is correlated with release of pro-inflammatory cytokines leading to increased apoptosis.

1.4.3 Th17 cells

Th-17 cells are also referred to as $CD4^+$ IL-17 producing T-cells (Dong, 2008). Low risk MDS is associated with a pro-inflammatory milieu where an increase in the levels of IL-17, IL-12, RANTES and IFN-γ were observed (Kordasti et al., 2009). In contrast, high amounts of the anti-inflammatory cytokine IL-10 and its soluble IL-2R were noted in high-risk stages (Kordasti et al., 2009). A high ratio of Th-17 cells to Tregs was demonstrated in low risk MDS and the inverse is true for the high-risk form, showcasing an inverse correlation between Th-17 cells and Tregs (Kordasti et al., 2009).

III. The complement systems

1.1 Introduction

The complement system is one of the oldest and most important components of innate immunity. It is often regarded as the first line of immunological defence and is a component of the innate immune system (Bajic et al., 2015), which does not change over a person's lifetime (Janeway et al., 2001). The complement system is deemed an effector arm of innate immunity, steering the identification and destruction of microbes in body fluids (Bennett et al., 2017).

Since its discovery in 1896 by Bordet, complement was for a long time associated with innate immunity until increasing evidence of the interplay between innate and adaptive immunity shed light on the contribution of complement to both innate and adaptive immune responses (Lambris et al., 2008, Zipfel et al., 2007, Garcia et al., 2016). Complement system comprises more than 50 circulating and membrane-bound proteins (Bajic et al., 2015). Most soluble components of the complement system are synthesized by the liver, although the occurrence of extrahepatic complement biosynthesis is also seen in other cell types (fibroblasts, T and B cells, adipocytes and endothelial cells), wherein the complement components are transported as inactive pro-proteins (Bennett et al., 2017, Morgan and Gasque, 1997).

1.2 Complement Components, Receptors and Regulators

1.2.1 CD46

CD46 (also known as membrane cofactor protein, MCP), a complement receptor on T-cells, plays a key part in modulating T-cell responses. CD46 is a transmembrane complement regulatory protein and serves as an inhibitor of complement activation on host cells and protects host tissue against unwanted complement deposition by functioning as a cofactor in the factor I- mediated degradation of C3b/C4b (Riley-Vargas et al., 2004). In humans, CD46 is expressed on all nucleated cells in four major isoforms (BC1, BC2, C1 and C2) that arise

through alternative splicing of a single gene (Figure 1-1). The N-terminus of CD46 consists of four so-called 'short consensus repeats' (SCRs) that contain the complement control function. This is followed by a heavily O-glycosylated region (differentially spliced B and C), a region of unknown function, a transmembrane anchor and one of two possible cytoplasmic domains, CYT-1 or CYT-2. Besides its role as a complement regulator, CD46 is also involved in the regulation of T-cell responses (Riley-Vargas et al., 2004). Activation of human primary $CD4^+$ T-cells in the company of CD46 cross-linking antibodies induces the development of a definite immunomodulatory T-cell population characterized by high IL-10/granzyme B production (Kemper and Atkinson, 2007). This response depends on signalling events mediated by cytoplasmic tails of CD46. In addition, CD46 is a receptor several important human pathogens including bacteria and viruses (Cattaneo, 2004) **(Figure 1-1)**. CD46 is involved in the down-modulation of adaptive Th1 immune responses by the regulation of IFN-γ and IL-10 production in the T helper cells (Cardone et al., 2011). Deficiency of CD46 is seen as a predisposing factor for many diseases causing complement-mediated 'self-attack' (Nesargikar et al., 2012).

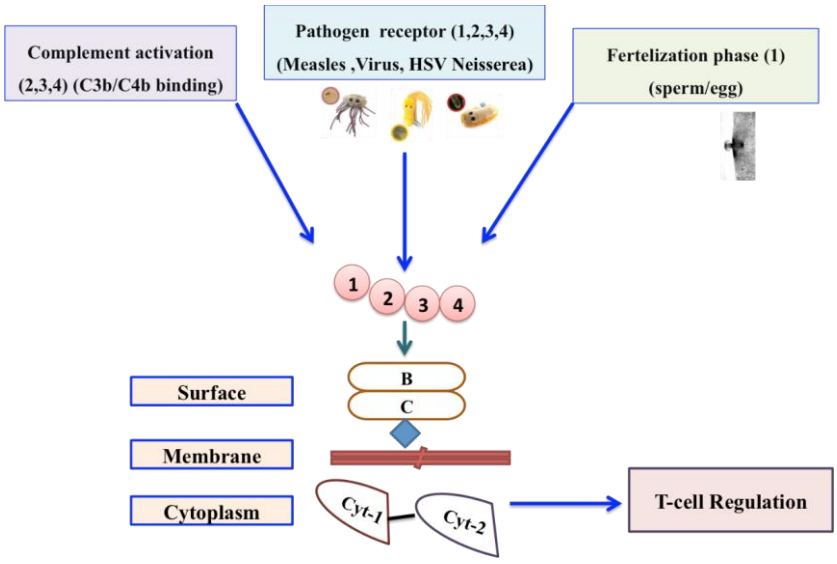


Figure 1-1: CD46, Structure and functions. CD46 is a complement regulatory protein
In humans, CD46 is expressed as four major isoforms (BC1, BC2, C1 and C2) on all nucleated cells. All four isoforms express short consensus repeats (SCRs) 1-4 on their N-terminus, followed by an alternatively spliced B or C region, transmembrane region and one of two possible tails, Cyt-1 or Cyt-2.

1.2.2 C3a and C5a and their receptors (C3aR, C5aR1, C5aR2)

The anaphylatoxins C3a and C5a are released when the convertases cleave C3 and C5 respectively and exert their biological functions after binding to seven-transmembrane domain (7TM) receptors in the membranes of host cells. Two of these receptors, C3aR and C5aR1 (CD88), are G protein-coupled receptors (GPCR), the third, C5aR2 (formerly known as C5L2), is structurally like C5aR1 but does not couple to heterotrimeric G proteins (Li et al., 2013). C5aR2 was first sought as a decoy receptor (does not undergo ligand-induced internalization), limiting the availability of the C5a and C5 adesArg ligands to C5aR1. C5aR2 may reduce the cellular responses to pro-inflammatory molecules and thus actively regulate inflammatory processes (Rittirsch et al., 2008).

Anaphylatoxins play an important role in chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and in asthma and allergy (Linton and Morgan, 1999, Woodruff et al., 2003, Hawlisch et al., 2004). C3a causes an anti-inflammatory response dependent on the cell type activated through C3a–C3aR signalling and the phase of inflammation (Coulthard and Woodruff, 2015). Anaphylatoxins are involved in tissue regeneration and development (Mastellos et al., 2001, Strey et al., 2003, Hillebrandt et al., 2005); in the liver, C3a induces STAT3 activation and an increase in IL-6 production (He et al., 2009); in the eye, C3a signalling activates STAT3 and promotes retinal regeneration (Haynes et al., 2013). C3a is further associated with neural stem cell migration and regeneration (Shinjyo et al., 2009), and C3aR has been implicated in alloreactivity (Asgari et al., 2013).

1.3 Complement and Adaptive Immunity (Intracellular Complement in T-Cells)

Activation of opposing functional phenotypes of $CD4^+$ T-cells is dependent on local complement **(Figures 1-2 A and B)** (Le Friec et al., 2013). The C3a and C5a that are locally produced bind to their receptors, functioning as autocrine and paracrine stimulators of T-cells and APCs (Soruri et al., 2003). Signalling through these G-protein-coupled receptors (GPCRs)

in T-cells leads to the activation of phosphoinositide-3-kinase-ã and induction of phosphorylation of phosphokinase B (AKT), upregulating the antiapoptotic protein (Bcl-2) and downregulating the proapoptotic molecule (Fas) (Le Friec et al., 2013, Soruri et al., 2003, Strainic et al., 2008). The expression of complement receptors C3aR and C5aR have been shown in peripheral, murine, natural regulatory T-cells (nTregs); signalling through these receptors inhibits Treg function (Le Friec et al., 2013). Mechanisms such as C3a/C5a-induced phosphorylation of AKT phosphorylate, the transcription factor Foxo1, result in lowered expression of Treg and Foxp3 (Le Friec et al., 2013). The co-stimulatory molecule signalling by CD28/CD80/CD86 and CD154/CD40 together minimize cell surface-expressed DAF, thus hampering complement activation, consequently resulting in the above changes (Cravedi and Heeger, 2015). DAF-deficient APC-induced T-cell expansion is seen to increase with diminished T-cell apoptosis (Lalli et al., 2008).

The proliferation of T-cells is enhanced and T-cell apoptosis is diminished by complement-dependent mechanisms (Lalli et al., 2008). T-cell homeostasis also requires C3aR/C5aR signalling; deficiency in both receptors leads to spontaneous accelerated T-cell death both *in vitro* and *in vivo* (Cravedi et al., 2013). C3 or C3 fragments are not secreted in individuals with serum C3 deficiency. Th1 cell responses are further severely diminished; however, sufficient intracellular C3a can be generated, mediating T-cell survival. This supports the hypothesis that C3aR is multi-functional at particular cellular locations (Liszewski et al., 2013). It has been observed that C3-deficient patients (in early childhood), CD46- deficient patients (throughout life) have greatly reduced Th1 cell- mediated responses (Th2 cell responses are normal) as well as a recurrence of infections, suggesting a difference in the complement-mediated signalling pathways contributing to the induction of Th1 cells in mice and humans (Ghannam et al., 2008, Le Friec et al., 2012). The results and findings largely portray, with regards to T-cell biology, a functional role of complement activation (Dunkelberger and Song, 2010). At the organism level, adequate evidence supports a relationship between complement activation and augmented T-cell immune response (Dunkelberger and Song, 2010).

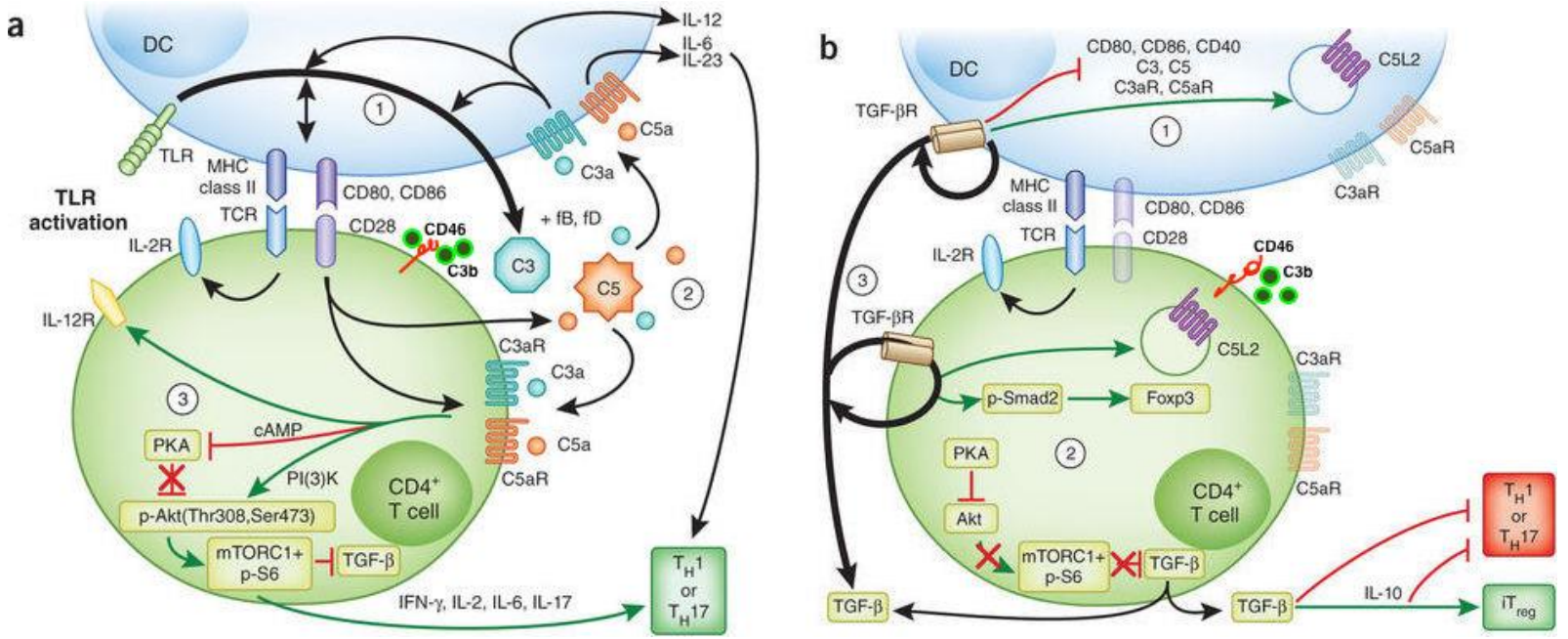


Figure 1-2: A and B: Opposing functional phenotypes of CD4$^+$ T cells dependent on activation in the presence or absence of local complement.

(A) The production of anaphylatoxins locally and induction of effector responses in Th1 or Th17 cells results from DC activation via the Toll-like receptor (TLR) engagement. Stimulatory molecules, major histocompatibility complex (MHC) class II, CD80 and CD86, as well as C3aR and C5aR, are upregulated by activated DCs, which also secrete components C3, C5, fB (factor B) and fD (Factor D) and downregulate CD55 (1). A functional C3 convertase is formed by C3, fB and fD under this circumstance, leading to local generation of C3a and C5a (2). C3a and C5a 'loop back' onto the DC by engaging with their respective receptors C3aR and C5aR, the expression of costimulators are sustained and the secretion of IL-6, IL-12 or IL-23 are induced (2). For the T cell, signals mediated by CD28, a costimulatory molecule, induce C3aR and C5aR expression, and lowered C3, C5, fB and fD secretion. The engagement of DC-derived C3a and C5a with their respective receptors on the T cells, induce IL-12R expression, activate phosphatidylinositol-3-OH kinase-ã (PI(3)K), inhibit PKA via cAMP generation inhibition, which consequently enables phosphorylation of Akt at Thr308 and Ser473 (p- Akt(Thr308,Ser473)) and ensues activation of mTORC1 and S6 (phosphorylation of S6 (p- S6)) (3) which are necessary for IFN-γ and IL-2 production. The balance is then tipped between Th1 and Th17 responses by DC-derived IL-6, IL-12 or IL-23, acting as cytokine prompts (Le Friec et al., 2013).

(B) No activation of receptors for pathogen-associated molecular pattern (PAMP) on DCs results in Foxp3+ iTreg cell induction and no local generation of anaphylatoxins. Minimal expression of CD80, CD86, C3aR and C5aR and low secretion of C3, C5 and fB and fD are seen when DCs are activated under tolerogenic conditions (1). There is the initiation of TGF-β production with a positive feedback loop, and suppression of proinflammatory cytokine secretion. The lack of DC-secreted anaphylatoxins and hence lack of signalling via the receptors in T cells enables PKA activation, consequently inhibiting configuration of the mTORC1 complex, preventing production of IFN-γ and IL-2, releasing the 'constraint' on the production of TGF-β and inducing suppressive Foxp3+ iTreg cells (2). IL-6 secretion is suppressed by TGF-β production, resulting in phosphorylation of p-Smad2 (the TGF-βR signal transducer Smad2) and expression of the transcription factor Foxp3 and of the receptor C5L2 (2). Local C5a are hunted by the receptor C5L2, ensuring sustained absence of its receptor C5aR from engaging. Under these conditions, both DC and T cell-produced TGF-β reciprocally sustain suppressive or tolerogenic phenotypes in both cells (3) (Le Friec et al., 2013, Dong, 2008).

Project Aim

The main aim of this PhD project was to investigate the potential role of intracellular complement system in polarisation and function of $CD4^+$ T-cells in MDS.

Project objectives

The main objective of this thesis was to elucidate the role of intracellular complement components (C3a and C5a), regulators (CD46) and their receptors (C3aR, C5aR1 and C5L2) in the $CD4^+$ T-cells polirization and switching towards Tregs phenotype, which is one of the main factors in the progression of HR-MDS patients towards AML. Specifically, the research sub objectives were:

1. To investigate whether there are differences between MDS and healthy donors in terms of intracellular and surface complement receptors (C3aR, C5aR1 and C5L2) and complement component (C3a and C5a) and if so, any differential expression between low and high-risk disease. Also, any potential differences between HDs, LR and HR-MDS in response to complement pathway activator (mainly CD46).

2. The main signalling pathways for polarising $CD4^+$ T-cells which including mTOR, and TGF-β signalling and associated proteins were investigated following stimulation with anti-CD46 in both HDs and MDS patients.

3. To identify additional pathways which may differentially expressed in T-cells from MDS patients compared to HDs, Tconv and Tregs were stimulated with anti-CD46 (with or without complement components) followed by RNA extraction for gene expression analysis.

Hypothesis

T-cells play a pivotal role in the pathogenesis of MDS. It has been shown that Tregs are increased in high risk MDS and correlate with poorer prognosis whereas pro-inflammatory T helper 1 (Th1) and T helper 17 (Th17) T-cells are the main feature in the low risk disease. Considering the importance of intracellular complement machinery in the polarisation of $CD4^+$ T-cells, I hypothesised that the malfunctioning of this machinery is crucial in Treg expansion and preventing an effective immune response in higher risk MDS.

Chapter 2. Material and Methods

The materials and methods used in this study are described below.

2.1 Sample preparation and culture

2.1.1 Patient sample and Ethical approval

Blood samples were obtained from 22 healthy donors and 44 MDS patients (out of which 22 had low-risk and 22 had high-risk MDS) with a median age of 67 years (range 35-86 years).

Patients were risk classified according to the WHO and IPSS criteria. The WHO subtypes and IPSS risk groups of patients are summarized in **Tables 1-2 and 1-3 (Chapter 1).** Written informed consent was obtained from all patients, including healthy donors, in accordance with the Declaration of Helsinki, approved by the King's College London Hospital Research Ethics Committee, and licensed by the Human Tissue Authority HTA (License no. 11023). All donors gave full informed consent prior to sample collection. The data did not include any identification information from the patients and was handled only by the members of the research team. Specific information on the characteristics of the MDS samples is listed in **(Table 2-1).**

Table 2-1: Specific information on the characteristics of the MDS samples

Sample	Cytogenetics	Molecular genetic data	r-IPSS score	% of BM blasts	Treatment date	Type of treatment	MDS (LR or HR)	Number of Vials
Sample 1	Normal	JAK2, CALR, MPL wild type	2.5	1	01/07/14	Epo	LR	2
Sample 2	Failed	Not Available	NA	1	20/01/14	Allo SCT	LR	2
Sample 3	Normal	Not Available	3	4	07/01/15	Allo SCT	LR	2
Sample 4	Normal	Negative for MGP	1	1	03/03/14	Lenalidomide	LR	2
Sample 5	Trisomy 8	SF3B1 mutated	3	1	01/07/16	W+W	LR	2
Sample 6	Normal	Not Available	2.5	1	14/10/13	Epo	LR	2
Sample 7	Failed	Not Available	3	1	01/07/13	Allo SCT	LR	2
Sample 8	Normal	Not Available	3	1	01/10/17	W+W	LR	2
Sample 9	Normal	Negative for MGP	1	1	31/03/15	Lenalidomide	LR	2
Sample 10	Normal	Not Available	2.5	1	02/10/17	Epo	LR	2
Sample 11	Normal	Negative for MGP	1	1	01/04/15	Lenalidomide	LR	2
Sample 1	Normal	Not Available	5.5	8	30/06/15	5 Aza	HR	2
Sample 2	Normal	RUNX1 mutated	5	15	02/02/15	Chemotherapy (DA 3+10)	HR	2
Sample 3	Normal	Not Available	6.5	12	01/08/12	5 Aza	HR	2
Sample 4	Trisomy 9	Not Available	7.5	18	25/04/13	5 Aza	HR	2
Sample 5	Normal	Not Available	6.5	14	09/03/16	Chemotherapy + Allo SCT	HR	2
Sample 6	Normal	Not Available	6.5	18	02/07/15	Allo SCT	HR	2
Sample 7	Normal	Not Available	6	22	01/09/15	Allo SCT	HR	2
Sample 8	Normal	JAK2, c-MPL wild type	3.5	5	01/11/15	Chemotherapy (DA 3+10)	HR	2
Sample 9	Normal	Not Available	5	7	01/12/15	5 Aza	HR	2
Sample 10	Trisomy 4	Not Available	5	4	01/10/15	LD Ara-C	HR	2
Sample 11	Normal	Not Available	4.5	21	06/04/11	5 Aza	HR	2

2.1.2 Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were isolated using a Ficoll-based gradient cell separation technique as follows: 15 ml of citrate anti-coagulated blood was taken from each donor and was carefully added onto 15 ml of Ficoll-Paque medium (GE Healthcare Life Science, Piscataway, New Jersey, USA) in a 50 ml Falcon tube (Greiner Bio-one, Chicago, USA), and then centrifuged at 450 g for 20 minutes at room temperature, with the brake off. Three layers were observed, with the layer of PBMCs (thick /buffy coat) lying between a layer of plasma and a layer of Ficoll-Paque medium. After aspiration using a sterile Pasteur pipette, the PBMCs were transferred into 50 ml Falcon tubes, washed twice with Hanks' Balanced Salt Solution (HBSS) with phenol red (Sigma-Aldrich, London, UK) in a final volume of 50 ml and centrifuged at 400 g for 5 minutes (brake on). After each wash, the supernatant was discarded and the final cell pellet was re-suspended in 8 ml of R10 medium comprising RPMI 1640 (Sigma-Aldrich, London, UK) supplemented with 10% fetal bovine serum (FBS) (50 ml) (Sigma-Aldrich, London, UK), and 5 ml of 100% (1x penicillin/streptomycin at a working concentration of 100,000 I.U.), penicillin/100,000 g/ml streptomycin per litre (Sigma-Aldrich, London, UK)).

Cells were counted using a haemocytometer and the density of cells was adjusted by the addition of medium to achieve a plating density of 1 million cells/ml cultured in 6-well tissue culture-treated plates (VWR international ltd, UK) or tissue culture flasks (VWR international ltd, UK). The cells were grown in an incubator at 37°C in humidified air containing 5% CO_2.

2.2 Peripheral blood mononuclear cell (PBMC) activation

The PBMCs were activated for 24 h in 48-well culture plates (2.0 - 5.0 x 10^5 cells/well) left uncoated or coated with mAbs to CD3 (2.0 g/ml), CD3 (2.0 g/ml) + CD28 (3.0 g/ml) or CD3 (2.0 g/ml) + CD46 (2.0 g/ml). The cells were grown in an incubator at 37°C in humidified air containing 5% CO_2.

2.2.1 $CD4^+$ T-cell isolation and activation

The PBMCs were separated using a ficoll-based gradient cell separation technique, as discussed in (2.1.2 section), and the $CD4^+$ T-cells were isolated using the MACS Human $CD4^+$ T-Cell Isolation Kit (Miltenyi Biotec, order no. 130-091-301, USA) following the manufacturer's instructions. $CD4^+$ T-cells were activated in 48-well culture plates (2.0-5.0x10^5 cells/well) left uncoated or coated with mABs to CD3 (2.0 µg/ml) alone, Anti-CD3 (2.0 µg/mL) + anti-CD28 (3.0 µg/mL) or anti-CD46 (2.0 µg/mL) antibodies were used to stimulate cells. Cells were untreated or treated with a combination of C3a/C5a (50 nM each). The cells were incubated for 24 hr at 37°C in a humidified atmosphere containing 5% CO_2. After culturing for 24 hr, 48-well culture plates were agitated gently to remove loosely adherent cells before analysis.

2.2.2 $CD4^+CD25^-$ (T-conv.) and $CD4^+CD25^+$ (Treg.) cells isolation and activation

The PBMCs were separated using a ficoll-based gradient cell separation technique, as discussed in (2.1.2 section). $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were isolated using the MACS Human $CD4^+CD25^+$ Treg Cell Isolation Kit (Miltenyi Biotec, order no. 130-091-301, USA) following the manufacturer's instructions. Purity of the isolated lymphocyte fractions was >95%. $CD4^+$ T-cells were activated in 48-well culture plates (2.0-5.0x10^5 cells/well) left uncoated or coated with mABs to CD3 (2.0 µg/ml) alone, Anti-CD3 (2.0 µg/mL) + anti-CD28 (3.0 µg/mL) or Anti-CD3 (2.0 µg/mL) + anti-CD46 (2.0 µg/mL) antibodies. Then cells were untreated or treated with a combination of C3a/C5a (50 nM each). The cells were incubated for 24 hr at 37°C in a humidified atmosphere containing 5% CO_2. After culturing for 24 hr, 48-well culture plates were agitated gently to remove loosely adherent cells before analysis. Cells were centrifuged at 300 g for 10 minutes and the supernatant were discarded. The cell pellet was re-suspended in 1ml Triazol and the vials were kept in -80 before using over the night.

2.3 Cryopreservation of cells

A 'Mr. Frosty' freezer container was pre-cooled in the fridge at 4°C and 1.8 ml sterile cryovials were labelled with the LSL number (sample identifier), name, date and cell number. After harvesting, the cells were centrifuged at 300 g for 10 minutes. After disposal of the supernatant, the cell pellet was re-suspended in a freezing mix, the serum-free and commercially prepared Synth-a-Freeze (Life Technologies, Order number A1254201), using 1 ml of the freezing mix per 5-10 x 10^6 PBMCs. The re-suspended cell pellet was aliquoted, 1 ml per vial, placed in a 'Mr Frosty' container (pre-cooled at 4°C) and frozen at -80°C overnight before being transferred to liquid nitrogen for long-term storage in vapour phase.

2.4 Thawing of cryopreserved cells

Cell cryovials from liquid nitrogen were thawed in a 37°C water bath for the minimum period necessary, then gently added drop-by-drop into 10 ml of pre-warmed R10 and centrifuged at 300 g for 10 minutes. The cells were washed twice with pre-warmed R10. After disposal of the supernatant, the cell pellet was re-suspended in 3 ml R10 (the volume of the media being dependent on the pellet size and cell number) and cells were counted and seeded in 6 well tissue culture-treated plates or tissue culture flasks. The cells were grown in an incubator at 37°C in humidified air containing 5% CO_2.

2.5 Cell counting

A mixture of 10µl of cell suspension and 10µl trypan exclusion was transferred into the counting chamber and all cells in the central large square of the haemocytometer were counted. The total number of cells was calculated: Cell count (25 sq grid) x dilution factor x 10^4 x volume of the cell suspension. To assess the viability of cells, trypan blue dye was used in the ratio of 1:1 and the total calculated cell number was multiplied by 2.

2.6 Cell staining

2.6.1 Gating and Compensation

Before running the samples, compensation adjustment was carried to prevent cross laser channels and the overlap of the colours, using BD CompBeads (BD Biosciences). The proper laser voltages were verified by running the setup beads (BD Biosciences), using FACSDiva software (BD Biosciences). These procedures ensured the consistency of laser readings throughout the entire project. The stained samples were run on the FACS machine after initial checks and compensation and started by 1 minute of acquisition at low speed to identify the targeted population followed by data recording of interested events. FACSDiva software (BD Biosciences) and Flow Jo software (Tristar, USA) were used to produce FACS plots for the thesis.

2.6.2 Surface staining

The PBMCs were harvested from the culture wells. Cell viability was monitored using Trypan blue and examination by light/optical microscopy. 2.0 x 10^5 to 1.0 x 10^6 cells were used per test for surface staining. Harvested cells were then transferred to pre-labelled FACS tubes (VWR, Chicago, USA). Alternatively, suspension cells were collected directly into FACS tubes for staining. In each case, cells were centrifuged at 300 g for 10 minutes. 500µl of supernatant was collected for later measurement of cytokine content. After removing the traces of supernatant, cells were re-suspended in 1 ml of phosphate buffered saline (PBS) only (without proteins). 1 µl of the live/dead efluor 780 dye (eBioscience Cat. No. 650865) was added to the cell pellet. Cells were then incubated for 30 minutes in the dark at room temperature (RT) and then washed twice by adding 1 ml of cold PBS and centrifuged at 300 g for 10 minutes. Specific antibodies (anti-CD4, anti-CD3, anti-CD25, anti-CD45RA and anti-CD127, anti-C3aR and anti-C5aR1) or (anti-CD4, anti-CD3, anti-CD25, anti-CD45RA and anti-CD127, anti-CD46 and anti-C5aR2 (C5L2)) antibody (4µl per 1 x 10^6 cells) were then added and topped up with cold PBS to final volume of 100 µl. Capped tubes were mixed by pulse vortexing followed by incubation in the dark at RT for 30 minutes. Following a further

wash in 2ml of cold PBS, cells were centrifuged at 300 g for 10 minutes. The supernatant was carefully aspirated from the tubes. After washing, the pellets were loosened by pulse vortexing the capped tubes. 1ml of freshly prepared eBioscience Fixation/Permeabilisation solution was added. The solution was added slowly, and the capped tubes were pulse vortexed and incubated for 30-60 minutes in the dark at 4°C before adding 2ml of 1x eBioscience Permeabilisation buffer directly to the tubes (total volume 3 ml) and cells were centrifuged at 300 g for 10 minutes. The supernatant was aspirated carefully, and the pellets were re-suspended by vortexing, washed again with 2 ml of 1x eBioscience Permeabilization buffer and centrifuged at 300 g for 10 minutes. 4µl of FOXP3 antibody was added and incubated for 30 minutes in the dark at 4°C. The cells were washed again with 2 ml of 1x eBioscience Permeabilization buffer and centrifuged at 300 g for 10 minutes. The supernatant was aspirated carefully, and the pellets were re-suspended by pulse vortexing. 200 µl of FACS buffer (PBS with 1% FCS) was added and the cells re-suspended to obtain a single cell suspension. Bound antibodies were then quantified by flow cytometry using a FACS Canto II or Fortessa Flow Cytometer.

2.6.3 Intracellular staining (IC)

Intracellular staining (eBioscience, Cat. No. 00-5123) was used for the detection and analysis of intracellular complement system proteins following the manufacturer's instructions. The steps of intracellular staining are similar to that of surface staining except where noted in the protocol; anti-C3aR and anti-C5aR1, anti-CD46 and anti-C5aR2 (C5L2) antibodies (4µl of each) were added with FOXP3 step.

2.7 Reagents

The reagents which were used in this study are described below:

Table 2-2: The two antibody panels used for the compensation and surface/intracellular antibodies cocktail for $CD4^+$ T-cells.

First Panel	Second Panel
C5aR1 PE (4µl/10^6)	CD46 PE (4µl/10^6)
C3aR APC (4µl/10^6)	C5L2 (C5aR2) APC (4µl/10^6)
CD3 V500 (4µl/10^6)	CD3 V500 (4µl/10^6)
CD4 efluor 450 (4µl/10^6)	CD4 efluor 450 (4µl/10^6)
L/D efluor780 (4µl/10^6)	L/D efluor 780 (4µl/10^6)
CD25 BV650 (4µl/10^6)	CD25 BV650 (4µl/10^6)
CD127 PerCp/Cy5.5 (4µl/10^6)	CD127 PerCp/Cy5.5 (4µl/10^6)
CD45RA Alexa Fluor 700 (4µl/10^6)	CD45RA Alexa Fluor 700 (4µl/10^6)
FOXP3 FITC (4µl/106)	FOXP3 FITC (4µl/106)

Fluorochrome antibodies were used for the fluorescence-activated cell (FACS) analysis as follows (Table 2-3):

Table 2-3: The antibodies and reagents used for the compensation and surface/intracellular antibodies cocktail for $CD4^+$ T-cells.

Antibodies and reagents	Manufacturer
Anti-human PE CD46	Biolegend, CA, Cat. No. 352402
Anti-human PE C5aR1	Biolegend, CA, Cat. No. 344304
Anti-human APC C5aR2 (C5L2) antibody	Biolegend, CA, Cat. No. 342406
Anti-human APC C3aR	Biolegend, CA, Cat. No. 345806
Anti-human PerCp/Cy5.5 CD127	eBioscience, CA, Ref. No. 45-1278-42
Anti-human BV650 CD25	BioLegend, CA, Cat. No. 302634
Anti-human FITC FOXP3	eBioscience, CA, Ref. No. 11-4776- 42
Anti-human Alexa Fluor 700 CD45RA	BioLegend, CA, Cat. No. 304120
Human CD3-VG™	MACS, mitenyi Biotechnology, UK, order No. 130- 098-164
Anti-human efluor®450 CD4	eBioscience, CA, Ref. No. 48-0049-42
Recombinant human complement component C3a	R&D Systems, Cat. No. 3677-C3
Recombinant human complement component C5a	R&D Systems, Cat. No. 2037-C5/CF
Live/Dead efluor780 dye	eBioscience Cat. No. 650865
UltraComp eBeads	eBioscience, CA, Ref. No. 01-2222-42

Table 2-4: Tissue culture reagents and equipment:

Reagents and Materials	Manufacturer
Roswell Park Memorial Institute medium (RPMI-1640)	Sigma Aldrich
Fetal Bovine Serum (FBS)	Sigma-Aldrich
Penicillin/Streptomycin/Ciprofloxicin	
L-Glutamine-Penicillin – Streptomycin solution	Sigma-Aldrich
Phosphate Buffered Saline (PBS)	PPA
15ml & 50ml Falcon tubes	VWR international ltd
Vented tissue culture flasks	
5ml, 10ml & 20ml Pipettes	
6,12, 24, 48 and 96 well plates	
1.5 ml microcentrifuge 'Eppendorf tubes'	Starlabs
Trypan Blue 0.4% , 0.85% NaCl	Lonza
Dimethyl sulfoxide (DMSO) (freezing cells)	Sigma
Neubauer Improved Haemocytometer	VWR international

Table 2-5: Purified Complements

Purified Complements	Clone	Manufacturer
Anti-human Purified CD46	TRA-2-10	Biolegend, CA, Cat. No. 352404
Anti-human Purified CD3	----------	eBioscience, CA, Ref. No. 16-0037-85

2.8 Flow cytometry analysis

Flow cytometry was used to analyse the activation of $CD4^+$ T-cells in the PBMCs and was also used to measure the level of surface and intracellular expression with stained markers. Cells were identified by the placement of gates using forward versus side scatter dot plots that had earlier been optimized to detect T-effector cells and T-regulatory cells as appropriate. Changes in fluorescence intensity resulting from the binding of fluorescently labelled antibodies to cells were expressed as mean fluorescence. A minimum of 10,000 events was collected per sample. Data were analysed using the FACS Canto II or the Fortessa Flow Cytometer.

2.9 Western blotting

Samples and controls were separated by SDS-PAGE under reducing conditions and transferred onto membrane.

2.9.1 Cell lysis

Samples of $2.5x10^5$ - $1x10^6$ $CD4^+$ T-cells were collected in 1.5 ml eppendorf tubes (Physiocare eppendorf AG, Hamburg, Germany) and centrifuged at 300 xg (Bifuge Pico-heraeus, Slough, UK) for 3 minutes and the supernatants were discarded. The tubes were centrifuged again at 300 xg for 30 seconds to remove the traces of media were removed. The dry pellets were flicked to disaggregate the cells and re-suspended in 12.5-25 µl 1X Nupage LDS sample buffer (Invitrogen, cat# NP0007, USA) + 10% Dithiothreitol (DTT) (100mM) (Applichem GmbH, Ottoweg, Germany) depending on the cell count.

Following vigorous mixing, the samples were boiled (Techne®, Dri-block DB.3, UK) at 70° C - 90° C for 8 min prior to separation by SDS-PAGE (Nupage 4-12% Bis-Tris Gel 1.0 mm x 12 well, Invitrogen-novex by life technologies™, USA) to denature the proteins completely. Each tube was centrifuged at 13,000-x g for 5 minutes and the supernatant was utilized for SDS-PAGE Nu-polyacrylamide gel (4-12 % Bis- Tris Gel) separation.

2.9.2 Western blot analysis

Following protein extraction by SDS-PAGE, western blotting was carried out on the samples. Nu-polyacrylamide gel (4-12 % Bis-Tris Gel) was placed in the electrophoresis tank (Invitrogen-Novex Mini-Cell, by Life Technologies, USA), which was filled with 1x MES SDS running buffer (NuPage® by Life Technologies). Protein samples were loaded (6-10 µl) along with 6 µl Precision Plus Protein™ Dual Xtra Prestained Protein Standards ladder (2-250 KD) (Cat# 1610377, Bio-RAD, Canada) to determine the molecular weight (MW) of the proteins. Electrophoresis was carried out at a constant voltage (200V) at run time 45 minutes (MES Buffer) until the dye reached the bottom of the gel. Next, the NuPage Bis-Tris gel was placed under a nitrocellulose-coated nylon membrane (Amersham, Hybond ECL,

Buckinghamshire, UK) and then sandwiched between blot filter paper (Bio-RAD, USA) and nylon pads soaked in NuPage transfer buffer. Proteins separated in the gel were transferred onto the nitrocellulose membrane by cold electrophoresis at 30 V constant for 120 min. Actin was used as a loading control (anti-beta actin (1:1000), Abcam Ab8226, 1 mg/ml). After the transfer, the nitrocellulose membrane was blocked for 1 hour with 5 % (w/v) dried skimmed milk (Marvel, UK) diluted in 1% PBS- 0.1 % (v/v) Tween 20® (Fisher scientific BP337-500, USA) and rinsed with 1% PBS- 0.1 % (v/v) Tween20®. As primary antibodies, mouse monoclonal anti-C5a/C5a des Arg antibody (2952, ab11878) or mouse monoclonal anti-C3a/C3a des Arg antibody (K13/16, ab36385) were used in the (1:500) dilution indicated previously in 1% (w/v) dried skimmed milk, 1%PBS-0.1% (v/v) Tween 20®, and incubated in the rotator overnight at 4°C. Membranes were subsequently washed three times in 1% PBS-0.1% (v/v) Tween20® for 15 minutes. This was followed by incubation for 1 h with the (1:1000) goat anti-mouse IgG-HRP (Abcam ab205719) secondary antibody in 1% (w/v) dried skimmed milk and 1% PBS 0.1% (v/v) Tween 20® at RT. Membranes were washed three times in 1% PBS- 0.1 % (v/v) Tween20® for 10 minutes. Bound antibodies were detected by incubation with ECL Western Blot substrate (Thermo-scientific, USA) for 3-5 minutes. The membranes were then sealed in Saran wrap and exposed to hyper film ECL developed using film developer system (Konica Minolta SRX101A, UK).

Table 2-6: The buffers and reagents used for the western blot

Reagents	Preparation
1X PBS	10 tablets were dissolved in 1 litre of dH_20
1X sample buffer	20 % (v/v) NuPAGE LDS Samples Buffer (4 x), 10 % (v/v) β-mercaptoethanol were made up in dH20 and stored at - 20°C.
NuPage Transfer buffer	50 ml 20X NuPage transfer buffer, 20 % Methanol (v/v), dH_20 upto 1 litre Buffer made by 1: 20 dilution of 20X Running buffer in dH20.
1x MES SDS Running buffer	The 1X Solution composed of 50 mM MES, 50mM Tris Base, 0.1 % SDS and 1mM EDTA with pH7.
RNaseA	10 mg/ml dH2O stored at -20°C
Blocking solution	5 % (w/v) Marvel milk powder was dissolved in 1X PBS buffer

Table 2-7: The antibodies used for the western blot

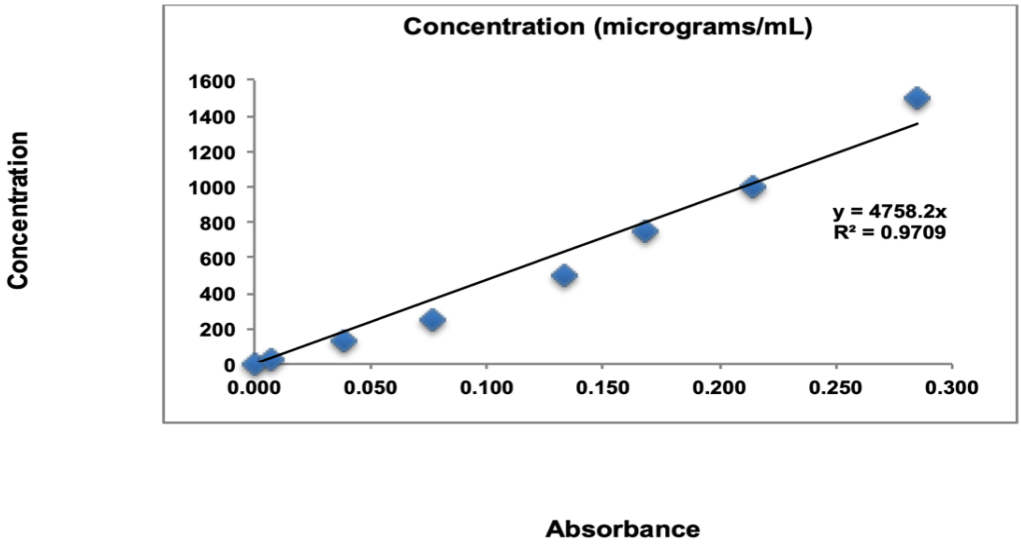
Primary	**Dilution**	**Manufacturer**
Mouse monoclonal anti-C3a/C3a des Arg antibody	1:500	Abcam ab36385
Mouse monoclonal anti-C5a/C5a des Arg antibody	1: 500	Abcamab11878
β-Actin	1: 1000	Abcam ab8226
Secondary	**Dilution**	**Manufacturer**
Goat anti-mouse IgG-HRP	1:1000	Abcam ab205719

2.10 Total protein extraction (BCA Protein Assay) and quantification by colorimetric methods

The total protein was extracted by bicinchoninic acid (BCA) and quantified using nano-drop. The Pierce BCA Protein Kit (Thermo Scientific Cat. 23225 sufficient for 500 tests) was used for total protein extraction following the manufacturer's instructions. The procedure involves the lysing of cells for extraction of the total protein using RIPA buffer and the BCA Protein kit for its quantification by colorimetric methods using a Nano-Drop spectrophotometer and measured as the absorbance at 540-562 nm. The BCA Protein kit is provided with a standard bovine serum albumin (BSA) solution of a known protein concentration. The kit allows the preparation of a series of dilutions to generate a standard curve of absorbance against protein concentration. Finally, the concentration of each unknown sample was determined based on the standard curve generated **(Table 2-8).**

Table 2-8: Standard curve (concentration of protein in µg/mL versus absorbance)

Absorbance	Concentration (micrograms/mL)
0.000	0
0.007	25
0.038	125
0.077	250
0.133	500
0.168	750
0.214	1000
0.285	1500



2.10.1 Protein extraction Reagents

1. Culture and thawing media (sterile): RPMI with 10% FCS and 1% Penicillin/Streptomycin. 5 ml P/S stock aliquots (100x, 10,000 units/ml penicillin, 10mg/ml streptomycin) were kept at -20°C. Once defrosted, they can be stored at 4°C for up to one month.

2. Phosphate buffered saline (PBS), with Calcium (II) and Magnesium (II). Bottles were kept sterile by working with only the aliquots taken inside the hood.

3. RIPA lysing buffer kit (Santa Cruz SC-24948). Aliquots containing 1.5mL were stored at -20°C. Aliquots were thawed as needed. There are other providers and the preparation of the buffer might be different. The kit contained PMFS, sodium ortho-vanadate in water, a protease inhibitor and 1x lysis buffer.

4. BCA Reagent A (1000 mL): containing sodium carbonate, sodium bicarbonate, bicinchoninic acid (BCA) and sodium tartrate in 0.1 M sodium hydroxide.

5. BCA Reagent B (25 mL): containing 4% cupric sulphate.

6. Working reagent (WR) diluent preparation: Total WR volume: 2400 µL (Stds) + 40 µL (9 Stds + Number of unknown samples). For example, for 4 unknown samples, the following was prepared: 2400 + 40 x (9 + 4) = 2920 µL (5 mL prepared). WR is 50 parts of BCA Reagent A plus 1 part of BCA Reagent B. Therefore, for 5 mL: 5 mL BCA A plus 0.1 mL BCA B (mixed until it became green in colour).

2.10.2 Cell lysis procedure

After 24 hr of incubation, the $CD4^+$ T-cells were harvested from the culture wells. 2.0 x 10^5-1.0 x 10^6 cells were used per test for the total protein extraction. Harvested cells were then transferred to pre-labelled Eppendorf tubes (for cell lysis) (Fisher Scientific, Product code 15128344).

In each case, cells were centrifuged at 300 g for 10 minutes. 500µl of supernatant was collected for later measurement of cytokine content. After removing the traces of supernatant, cells were washed twice with ice cold phosphate buffered saline (PBS), then re-suspended in 250 µL of PBS (ice-cold) and pelleted in the micro-centrifuge at 3000g for 2 minutes, after which the supernatant was carefully removed. The complete RIPA buffer was prepared in the following way:

1 mL of complete RIPA buffer prepared for lysing up to 10^7 cells by mixing 10 µL of PMFS, 10 µL of sodium ortho-vanadate in water, 20 µL of protease inhibitor and 960 µL of 1x lysis buffer. Cells were re-suspended in lysing buffer (35 µL per tube), pulse vortexed to mix up and then cells were incubated by maintaining constant agitation for 30 minutes at 4°C (using ice box). The cells were pelleted in the micro-centrifuge for 20 minutes at 16000 g and 4°C to give the supernatant containing the total protein.

2.10.3 Preparing standard dilutions

The Pierce BCA Protein Kit was used to prepare the bovine serum albumin (BSA) diluted standards. One ampule contained 2 mg/mL BSA in PBS (top standard). For preparation of the standards, the instructions provided by the kit were followed: Micro-plate procedure (working range 20-2000 µg/mL) **(See Table 2-9).** 5 µL of each standard dilution or unknown sample was pipetted into wells in a 96-well plate. 40 µL of the working reagent (WR) was added to all wells and mixed by pipetting up and down several times or putting the plate on the shaker for 30 seconds. The plate was covered, incubated at 37°C for 30 minutes and left to cool down at room temperature (RT). The protein quantification was measured at 562nm absorbance using a Nano Drop spectrophotometer 2000/2000c. A standard curve (concentration of protein in µg/mL versus absorbance) was generated in an Excel spreadsheet and the protein concentrations in the samples were extrapolated from it (**Table 2-9).**

Table 2-9: Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent (microliters)	Volume and Source of BSA (microliters)	Final BSA Concentration (microliters/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G	25
I	400	0	0 = Blank

2.11 Luminex immunoassay (Total TGF-β Protein expressions of phosphorylated mTOR, PI3K/Akt, ERK, Smad2, Smad3, Smad4 and TGFRβII employing Luminex® technology)

The MILLIPLEX MAP Human TGF-β Signaling Magnetic Bead Panel 6-plex- Cell Signaling Multiplex Kit 96-well Plate Assay (Cat. # 48-614MAG) was used to detect the signalling changes in phosphorylated mTOR, PI3K/Akt, ERK, Smad2 (Ser465/Ser467) and Smad3

(Ser423/Ser425), as well as total protein levels of TGFRβII and Smad4 in cell lysates using the Luminex® system following the manufacturer's instructions. 25 ug of sample was used in each well and the immunoassay was carried out according to the published protocol for this kit. Although the TGFβ pathway regulates a wide range of processes, the pathway is fairly simple. TGFβ dimers bind to a TGFβ Type II Receptor, which recruits and phosphorylates a TGFβ Type I Receptor. The Type I Receptor then recruits and phosphorylates SMAD2/3, which then binds to SMAD4 and forms a complex that enters the cell nucleus, where it acts as a transcription factor for various genes. The TGFβ Receptor activates the SMAD-dependent canonical pathway, as well as SMAD-independent non-canonical pathways, such as mTOR, PI3K/Akt, ERK, JNK, and p38. The data was acquired on a Luminex 200TM (Luminex Corporation, Austin, TX, USA). The Luminex 200™ instrument with xMAP® technology system and xPONENT® acquisition software were employed to capture component detection. Milliplex® Analyst 5.1 software was adopted to integrate data acquisition and analysis.

2.11.1 Luminex immunoassay protocol:

Day 1

A. Preparation of reagents

- **1x capture beads**

The 20x stock magnetic beads were sonicated for 15 seconds, vortexed for 30 seconds and then the beads were diluted to 1x by combining 150 µL (TGF-beta 6-plex) + 150 µL (mTOR MAPmate) beads with 2.7 mL of assay buffer 2 in mixing bottles. The 1x capture beads were further vortexed for 15 seconds.

- **Lyophilized cell lysate**

There were three vials provided in the kit: unstimulated Hela lysate negative control, HepG2 stimulated with TGFb 60 min lysate, and MCF7 stimulated with IGF-1 for 10 minutes. All vials were reconstituted by adding 100 µL ultra-pure water (final

concentration of protein 2 mg/L) and kept for 5 minutes at RT. 150 µL of AB2 were added to each bottle, vortexed and stored on ice till use.

B. Protocols

Samples were centrifuged at 10000 g for 5 minutes to bring any particles to the bottom. Wetting the plate was done by adding 50 µL AB2 and then shaking for 10 minutes. It was covered and sealed with autoclave tape to avoid splashes. The buffer was decanted in the sink, and dried well on paper towels. To each well the following were added: 25 µL 1x capture beads + 25 µL AB2 to the blank wells or control reconstituted lysates in duplicate or unknown samples (only one well per sample) and incubated overnight (16-20 hours) at 2-8°C on a plate shaker (600-800 rpm) protected from light.

Day 2

A. Preparation of reagents

- **Preparation of Biotin-Labeled Detection Antibody**

The 20x detection antibody stock was vortexed for 10 seconds, and centrifuged briefly after vortexing for complete recovery of contents. The detection antibody was diluted to 1x by combining 150 µL (TGF-beta 6-plex) + 150µL (mTOR MAPmate) biotin with 2.7 mL of assay buffer 2 (AB2) in mixing bottles. The 1x biotin was further vortexed for 15 seconds.

- **Preparation of Streptavidin-PE Detection Antibody**

This was provided as a 20x stock solution. The Streptavidin-Phycoerythrin 1:25 (SAPE) was vortexed for 10 seconds. SAPE was diluted by combining 120 µL of Streptavidin-Phycoerythrin with 2.88 mL of Cell Signalling Assay Buffer 2 using one of the mixing vials provided. The1X biotinylated detection antibody and SAPE were transferred with a pipette to separate reservoirs.

B. Protocols:

A handheld magnetic separation block was attached to the plate after removing it from the shaker, allowing 60 seconds for beads to settle and then decanting the samples and controls. After removing the plate from the magnetic separation block, it was washed with 100 mL Assay Buffer per well and repeated for a total of two washes. 25 µL/well of 1x Milliplex® Map Detection Antibody was added, the plate was sealed, covered with a lid and incubated with agitation on a plate shaker for 1 hour at room temperature (20- 25°C). The Magnetic Separation Block was attached, and after 60 seconds the Detection Antibody was decanted. 25 µL of 1x Milliplex® Map Streptavidin-Phycoerythrin (SAPE) was added, then the plate was sealed, covered with a lid and incubated with agitation on a plate shaker for 15 minutes at room temperature (20-25°C). The SAPE was not removed. 25 µL of Amplification Buffer was added to each well, the plate was sealed, covered with lid and incubated with agitation on a plate shaker for 15 minutes at room temperature (20- 25°C). The Magnetic Separation Block was attached, and after 60 seconds the SAPE/Amplification buffer was decanted. The beads were suspended in 150 µL of Milliplex® Map Assay Buffer, and mixed on a plate shaker for 15 minutes, then analysed using the Luminex® system.

2.12 Cytokine assay

To determine serum cytokine levels, cytokines were assayed in supernatants using the ProcartaPlex Human Cytokine and Chemokine 34-plex Antibody Bead Kit (ThermoFisher Scientific, Waltham, MA USA, Cat no: EPX340-12167-901) according to the manufacturer's instructions, and data were gathered by a Luminex 200TM (Luminex Corporation, Austin, TX, USA). Many cytokines, chemokines and related proteins were quantified with ProcartaPlex Human Cytokine and Chemokine 34-plex supplemented kit using a Luminex MagPix system and Luminex xPonent Software. The culture medium samples were assayed undiluted. Milliplex Analyst software (VigeneTech) was used for

the multiplex assay data extraction. The level of each cytokine was calculated from a standard curve derived from known controls. Supernatant samples were taken at 24 h time points before and after stimulation. Cytokines, chemokines and related proteins were identified as in the **(Table 2-10)**:

Table 2-10: List of cytokines, chemokines and related proteins quantified

Cytokines, chemokines and related proteins
Eotaxin
Granulocyte macrophage colony stimulating factor (GM-CSF)
Melanoma growth stimulatory activity alpha (GRO-α)
Interferon alpha 1 (FNA1)
Interferon gamma (FNG)
Interferon-gamma inducible protein-10 (IP-10)
Monocyte chemoattractant protein (MCP-1)
Macrophage inflammatory protein 1-alpha (MIP-1α)
Macrophage inflammatory protein 1-beta (MIP-1β)
Regulated on activation, normal T-cell expressed and secreted (RANTES)
Stromal cell-derived factor 1 alpha (SDF-1α)
Tumour necrosis factor-alpha (TNF-α)
Tumour necrosis factor-beta (TNF-β)
Interleukin (IL)-10, IL-12p70, IL-13, IL-15, IL-17A, IL-18, IL-1a, IL-1β, IL-1RA, IL-2, IL-21, IL-22, IL-23, IL-27, IL-31, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9

2.12.1 Cytokine Assay Protocol

A. Preparing test samples

Test samples were thawed just before use and spun down at 10000 g for 2 minutes to bring any impurities to the bottom of the tube. The appropriate volumes of samples (cell culture supernatants: 60 µL) were dispensed into a 96-well V-bottom plate, which was then covered (sealed with tape) and frozen down at -20^{o}C overnight for next day analysis.

B. Preparing buffers

- **Wash buffer (1x):** 200 mL dilute 20 mL of the stock (10x) bottle was prepared and added to 180 mL of deionized water in a glass bottle. This was allowed to warm up to room temperature before use. The wash buffer (1x) can be stored at 2-8 ^{o}C for up to six months.

- **Universal Assay Buffer (1x) (UAB):** This is provided at the right dilution in some kits, if not, then the instructions for that particular kit needs to be followed. The UAB (1x) can be stored at 2-8^{o}C for up to 30 days.

C. Thawing samples

The plate with samples was taken from the freezer and placed on ice to thaw.

D. Preparing standards

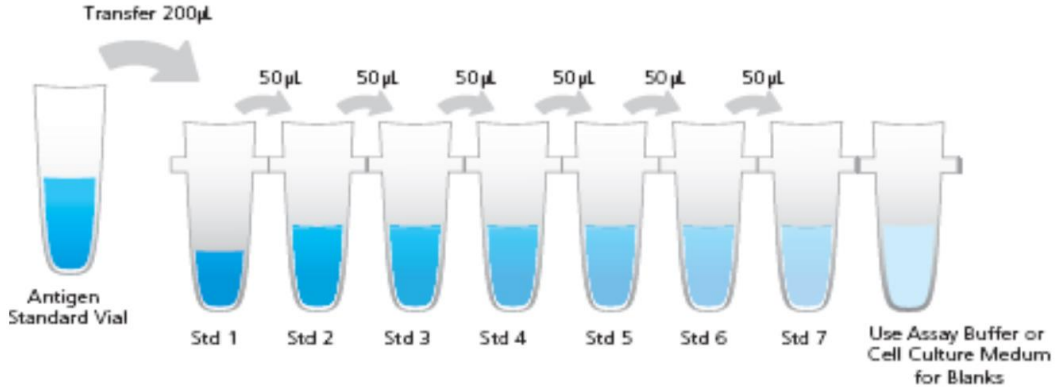
Each of the different antigen standard vials was centrifuged to mix A and B at 2000 x g for 10 seconds to collect the contents at the bottom of the bottles. 250 µL of UAB was dispensed in Standard Mix A bottle and the vials were gently vortexed for 30 seconds and incubated on ice for 5-10 minutes to give the Diluted Standard Mix A. 250 µL of the Diluted Standard Mix A was transferred into the Standard Mix B bottle and vortexed gently for 30 seconds, followed by incubation on ice for 5-10 minutes to give the Diluted Standard Mix B (DSM B). The remaining standards were prepared from serial dilution 1:4 with UAB of the DSM B (top standard) **(Table 2-11),** making sure to change the tips every time. Once prepared, the standard tubes were stored on ice until needed. 2 x 8 small Eppendorf tubes in a rack (standards are done in replicate) were prepared and 150 µL was added to tubes 2 to 7. Tube 1 was the top standard and 8 the blank. A 4-fold serial dilution was prepared of the reconstituted standards using the PCR 8-tube strip provided.

Tubes were labelled Std1, Std2, Std3, Std4, Std5, Std6 and Std7; then, 200 µL of the reconstituted antigen standards was added to the first tube, i.e. Std1 of the strip of tubes. 150 µL of sample type specific standard buffer was added to Std tubes 2-7. Universal Assay Buffer was used for serum or plasma samples and cell culture media for culture supernatant samples. 50 µL of the reconstituted antigen standards was transferred from Tube 1 into Tube 2, mixing by pipetting up and down for a total of 10 times. 50 µL of the mixed standards from Tube 2 was transferred into Tube 3 and mixed by pipetting up and down for a total of 10 times. This

was repeated for Std tubes 4-7. Next, 200 µL of Universal Assay Buffer or cell culture medium was added to tube 8, which served as a blank and was kept on ice until ready for use.

Table 2-11: Preparation of 4-Fold Serial Dilution

Standards	01	02	03	04	05	06	07	08
Dilution	Top standard	1/4	1/16	1/64	1/256	1/1024	1/4096	Blank
Volume (µL)	200 DSM B	50 Std01+ 150 UAB	50 Std02 + 150 UAB	50 Std03 + 150 UAB	50 Std04 + 150 UAB	50 Std05 + 150 UAB	50 Std06 + 150 UAB	150 (UAB only)



E. Define the plate map.

The standard, sample and blank wells were marked using the recommended and blank plate layout **(Table 2-12).**

Table 2-12: The plate map layout

Standards		Samples									
Standard 1	Standard 1	1	1	9	9	17	17	25	25	33	33
Standard 2	Standard 2	2	2	10	10	18	18	26	26	34	34
Standard 3	Standard 3	3	3	11	11	19	19	27	27	35	35
Standard 4	Standard 4	4	4	12	12	20	20	28	28	36	36
Standard 5	Standard 5	5	5	13	13	21	21	29	29	37	37
Standard 6	Standard 6	6	6	14	14	22	22	30	30	38	38
Standard 7	Standard 7	7	7	15	15	23	23	31	31	39	39
Blank	Blank	8	8	16	16	24	24	32	32	40	40

F. Preparing the capture bead mixture

The capture bead bottles were vortexed for 30 seconds. The flat-bottom 96-well black plate provided in the kit was installed on the Hand-Held Magnetic Plate Washer (HHMPW), making

sure it was securely attached (firstly the right side was installed followed by the left, and the mobile clip was adjusted to securely attach the plate onto the platform). 5 mL of each capture bead bottle provided in the kit was pipetted into a 50 mL tube. The capture bead mixture was transferred into a multichannel pipette reservoir. The appropriate amount of capture bead mixture was dispensed into each well of the plate. 50 µL was dispensed for each bead bottle used in the final mixture. Hence, for a kit containing 4 different bottles of magnetic beads, 4 x 50 = 200 µL was dispensed into each well of the plate. The black plate cover was placed to protect the beads from the light and 2 minutes were allowed for the beads to become magnetically attached to the bottom of the wells.

Making sure the plate was properly installed in the HHMPW, the liquid in the wells was discarded by quick inversion in the sink (one or two pulses) and the plate was blotted on a paper towel. The magnetic beads remained attached to the bottom of the wells. A new multichannel pipette reservoir was prepared with wash buffer (the reservoir was labelled using a marker pen). This reservoir could be washed and reused in further experiments. To wash the capture beads once, 150 µL of washing buffer was added to all the wells and the plate was covered to protect the beads from the light (cover was provided). The plate-HHMPW was rested on the lab bench for 2 minutes. Making sure the plate was properly installed in the HHMPW, the liquid in the wells was discarded by quick inversion in the sink (one or two pulses) and the plate was blotted on a paper towel. The magnetic beads remained attached to the bottom of the wells.

G. First incubation (capturing the cytokines)

Serum and plasma samples were diluted 1:2 by adding UAB to the wells (30 µL) and mixed up and down with the multichannel pipette. Standard and culture supernatant samples did not need diluting. 50 µL of all samples (diluted serum, diluted plasma and supernatants) were dispensed into the appropriate wells on the black plate. Duplicate analyses were done for the standards and single for the test samples. The plate was sealed with an adhesive slip (provided by the kit)

and covered to protect the beads from the light (cover usually provided, otherwise aluminium foil can be used). The plate was then detached from the HHMPW platform and incubated for 1-2 h in the shaker at room temperature in the dark. It was ensured that the plate was securely fixed to the shaker using autoclave tape. The plate was taken from the shaker and installed on the HHMPW platform, waiting 2 minutes to allow the beads to become magnetically attached to the bottom of the wells.

The seal was carefully removed and, making sure the plate was properly installed in the HHMPW, the liquid in the wells was discarded by quick inversion in the sink (one or two pulses) and the wells were blotted on a paper towel. The magnetic beads remained attached to the bottom of the wells. After washing the plate twice after the first incubation, 150 µL of washing buffer was added to all wells and the plate was covered to protect the beads from the light. The plate-HHMPW was rested on the lab bench for 30 seconds. Making sure the plate was properly installed in the HHMPW, the liquid on the plate was discarded by quick inversion in the sink (one or two pulses) and the plate was blotted on a paper towel. The magnetic beads remained attached to the bottom of the wells.

H. Second incubation (adding the biotinilated detection antibodies)

3 mL of the detection antibodies mixture (1x) was prepared: The bottles (4 bottles: B, C, D and F) were briefly spun down. In a 15 mL conical tube, 60 µL of each bottle was dispensed and 3000 – (60 x 4) = 2760 µL of detection antibodies diluent was added. This was gently vortexed and the detection antibodies mixture was transferred to a multichannel pipette reservoir. The plate was removed from the HHMPW platform and 25 µL of the mix was added to all wells without mixing (tips were changed each time). The plate was sealed with an adhesive slip (provided by the kit) and covered to protect the beads from the light. The plate was detached from the HHMPW platform and incubated for 30 minutes on the shaker at room temperature in the dark. The plate was securely fixed to the shaker using autoclave tape. After removing the plate from the shaker it was installed on the HHMPW platform for 2 minutes to allow the

beads to become magnetically attached to the bottom of the wells. The seal was carefully removed, and the plate was washed twice after the second incubation; 150 µL of washing buffer was added to all wells and the plate was covered to protect the beads from the light (cover usually provided).

The plate-HHMPW was rested on the lab bench for 30 seconds and, making sure the plate was properly installed in HHMPW, the liquid on the plate was discarded by quick inversion in the sink (one or two pulses) and the plate was then blotted on a paper towel. The magnetic beads remained attached to the bottom of the wells.

I. Third incubation (Adding Streptavidin-PE antibodies)

The SA-PE antibody was transferred to a new multichannel pipette reservoir and plate from the HHMPW platform. 50 µL SA-PE antibody was added to all wells without mixing and by changing the pipette tips every time.

The plate was sealed with an adhesive slip (provided by the kit) and covered to protect the beads from the light (cover usually provided, otherwise aluminium foil can be used). The plate was detached from the HHMPW platform and incubated for 30 minutes on the shaker at room temperature in the dark. The plate was securely fixed to the shaker using autoclave tape. After removing the plate from the shaker it was installed on the HHMPW platform for 2 minutes to allow the beads to become magnetically attached to the bottom of the wells. The seal was carefully removed, and the plate was washed twice after the third incubation; 150 µL of washing buffer was added to all wells and plate was covered to protect the beads from the light (cover usually provided).

The plate-HHMPW was rested on the lab bench for 30 seconds and, making sure the plate was properly installed in HHMPW, the liquid on the plate was discarded by quick inversion in the sink (one or two pulses) and the plate was then blotted on a paper towel. The magnetic beads remained attached to the bottom of the wells.

J. Preparing the plate for reading on the Luminex instrument

120 µL of reading buffer was added into each well. The plate was sealed with a new plate seal, covered with the black microplate lid and incubated 5 min on a plate shaker at RT at 500 rpm. After removing the seal the plate was run on a Luminex Instrument. The concentration of the samples was calculated by plotting the expected concentration of the standards against the MFI generated by each standard.

2.13 RNA preparation for microarray analysis

For preparation of microarray samples there are two methods of RNA purification that are commonly used. Method (I) is well established and is commonly used by Affymetrix users. Method (II) is a shortened derivative of Method (I) and has been used by MUSC DNA microarray users with good success.

- Method (I). This involved purifying the RNA using Trizol according to manufacturer recommendations. The resulting RNA was taken and purify the RNA further using Qiagen RLT buffer as this may increase GeneChip background.

- Method II. Trizol/RNeasy hybrid protocol. This method begins with Trizol homogenization and extraction. After this, the aqueous phase (containing the RNA) is combined with 70% ethanol and loaded onto an RNeasy column. Purification proceeds as described in Qiagen protocols (Cat No./ID: 74104, Germany). This method has the advantage of being simple and relatively short. It also limits handling of the RNA, perhaps reducing degradation.

Method (II) has been used in this project (Trizol/RNeasy hybrid protocol).

Adapted from communication by Alvydas Mikulskis, an appropriate volume of Trizol, Store homogenate at RT of 5 min.

2.13.1 Total RNA isolation

Cell pellets in the range of 10×10^5-1×10^6 cells were resuspended in 1 ml of Trizol (Invitrogen) and vortexed vigorously. These pellets were either stored at -80 ˚C or the samples were processed immediately by incubating at RT for 5 min. The homogenised samples were mixed with 200 µl of chloroform and then centrifuged at 12,000 xg for 15 min at 4˚C. The separated upper aqueous phase was transferred into a fresh tube. 500 µl of 2-Propanol was added to the aqueous phase and the mixture was incubated for 10 min at RT. The samples were centrifuged at 12,000 xg for 10 min at 4˚C. The supernatant was discarded, and the RNA pellet was washed with 1000 µl of 75% (v/v) ethanol at 7500 xg for 5 min. The supernatant was discarded, and the RNA pellet was air-dried for 10-15 min. The pellet was dissolved in 30 µl of RNase free water and the concentration was determined using Thermo Scientific Nanodrop sphectrophotometer. Alternatively, RNeasy Micro Kit - RNeasyMinElute Spin Columns were used for purification of concentrated total RNA when dealing with small numbers of cells as per manufacturer's instructions.

2.14 Gene Expression

(Jaksik et al., 2015), Provides a good overview of the Microarray Analysis from RNA extraction through to Data Processing. The microarray protocol is also described in Mesnage et al (2017). Below is a brief summary of the work;

RNA extraction was performed using the MagMAX (Thermo-Fisher catalog number: AM1839) and Agencourt RNAdvance Cell V2 kit (Part number: A47942) according to the manufacturer's instructions. The samples were checked for RNA quality using the Agilent 2100 Bioanalyzer Pico kit (Cat no: 5067-1513) and quantified using the Nanodrop (ND-1000 Spectrophotometer). Samples with RNA intergrity number (RIN) of higher than 7 were selected for the microarray study. 500 pg of total RNA was used per sample. Poly-A Spiking Control (Thermo-Fisher 900433) was used as an internal amplification control. SPIA cDNA was generated usingNuGenOvation Pico WTA System V2 kit (Cat.no:3302-60), following the

manufacturer's instructions. The SPIA cDNA was subjected to a quality control (QC) using the Agilent 2100 Bioanalyzer Nano kit (5067-1511) and quantified by Nanodrop ND-1000 Spectrophotometer. The 5ug of SPIA cDNA was fragmented and Biotin-labelled using the NuGen Encore Biotin Module (Cat. no: 4200-60) according to the manufacturer's instructions. The fragmented and Biotin-labelled cDNA was subjected to a further round of QC to assess fragmentation size (Agilent 2100 Bioanalyzer Nano kit; fragment size < 200nt).

Hybridization cocktails were prepared according to Nugen's recommendations for midi arrays (100 format) (Encore Biotin Module User Guide Appendix) and Human Gene 2.0 ST Arrays (Thermo Fisher, 902113) were hybridized at 45oC for 16-20 hours in an Affymetrix Genechip Hybridization oven 645 (Cat no: 0-0331). The arrays were washed and stained using wash protocol FS450_0002 as recommended by Affymetrix, (Thermo Fisher) on the GeneChip Fluidics station 450. The arrays were scanned using the Affymetrix GeneChip Scanner. CEL files were quality control assessed in the Expression Console software package (Affymetrix, Thermo FIsher) by using standard metrics and guidelines for the Affymetrix microarray system. Data were normalised together using the Robust Multi-array Average (RMA) sketch algorithm. The study was performed in 3 separate groups (roughly 28 x 3) (Mesnage et al., 2017).

Table 2-13: RNA extraction reagents

Reagents	Manufacturer
TRIzol	Invitrogen
RNase free water	Qiagen
Phase lock Gel Heavy 2 ml	5 PRIME
RNeasy Micro Kit	Qiagen
Chloroform	Sigma
Propanol	
Ethanol	Sigma

2.15 Expression microarray data analysis

Gene expression analysis was done under the guidance of Dr. Shahram Kordasti. The HT 2.0 arrays were scanned using a GeneChipR 3000 Scanner (7G upgrade) to capture images (DAT files). The raw data from the DAT file is converted to CEL files (based on intensities). ITERPLIER normalization was used to generate the CHP file. Ingenuity Pathway Analysis (IPA) was used to detect differential gene expression. In case of mutations, the gene expression in low-risk and high-risk MDS patients with the mutant was compared with that of healthy donors. The data was filtered using IPA p value ≤ 0.05 to create a list of significantly up or down regulated genes.

2.16 Statistical analysis

Data were entered into and analysed using Microsoft Excel and the results are presented as the mean ± standard deviation (SD). The data is normally distributed and analysis of variance between groups was performed using a parametric approach (one-way ANOVA and student T-tests). P-values of < 0.05 were considered statistically significant. All statistical analysis was performed using GraphPad prism 6.0. (GraphPad Prism version 6.0 for Mac, GraphPad Software, San Diego California USA, www.graphpad.com).

Chapter 3. Results I

Comparative study of Complement Components, Regulators and Receptors in Healthy Donors vs. MDS Patients

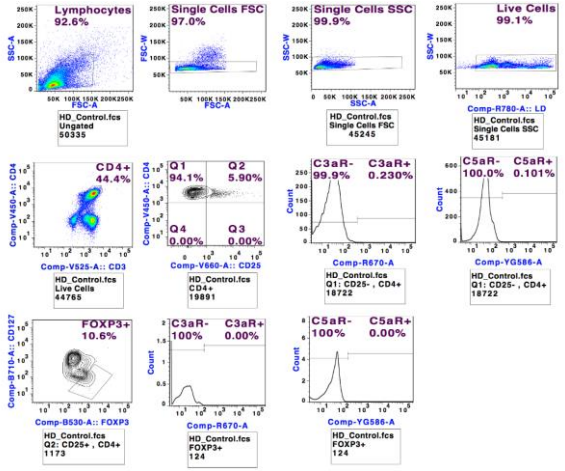
3.1 Introduction

In this chapter the intracellular and surface expressions of the following complement receptors (C3aR, C5aR1 and C5aR2, CD46) in Tconv and Treg $CD4^+$ T-cells from healthy donors, low-risk and high-risk MDS patients were measured by flow cytometry. The expressions of complement components (C5a and C3a) were determined by Western blotting (WB). The results suggest that insufficient or excessive function and/or distribution of complement expression may partly contribute to inappropriate $CD4^+$ T-cells activity in MDS.

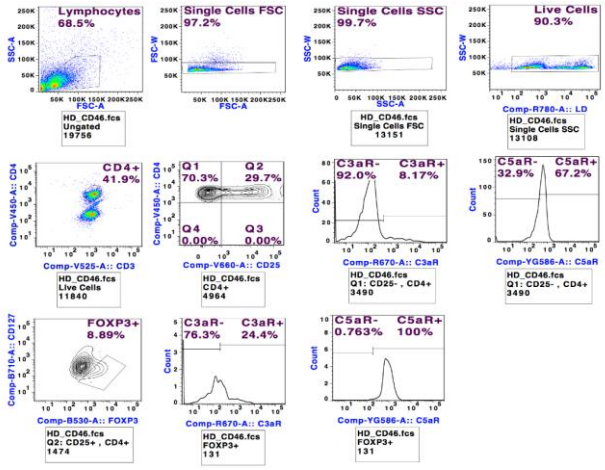
3.2 Gating strategy for flow cytometry analysis

The **Figures in 3-1** are from healthy, low-risk and high-risk MDS donors respectively; (**A, C and E)** representing cells from the control donors; i.e. non-activated, and (**B, D and F)** donors whose cells were activated with anti-CD3+CD46 antibody for 24 hours.

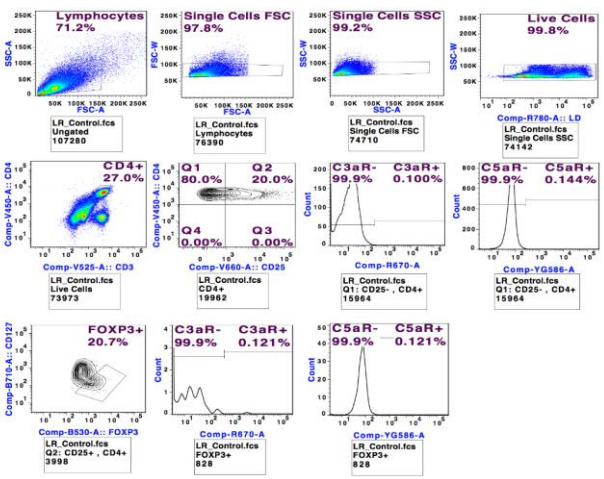
A: HD; non-activated



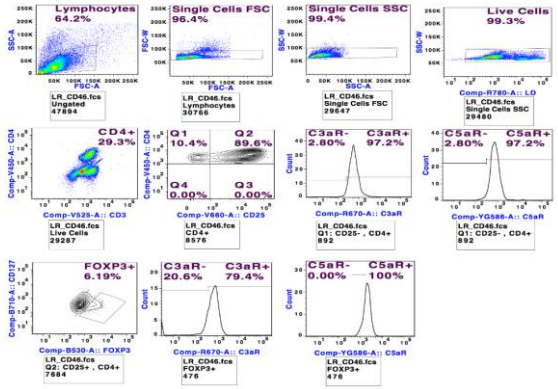
B: HD; activated with anti-CD46



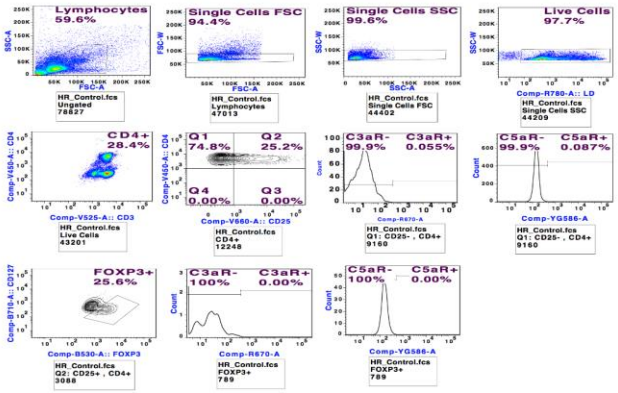
C: LR-MDS; non-activated



D: LR-MDS; activated with anti-CD46



E: HR-MDS; non-activated



F: HR-MDS; activated with anti-CD46

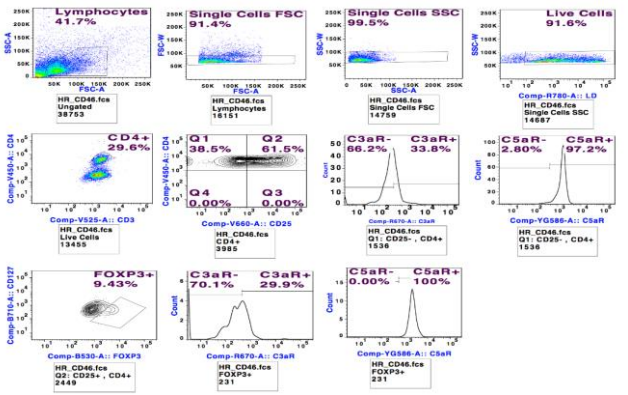


Figure 3-1: Showing the gating strategy in healthy donor (A and B), low-risk MDS (C and D) and high-risk MDS (E and F) donors. A, C and E cells were the control (non-activated) cells and B, D and F cells were activated with anti-CD46 antibody for 24 hours. The PBMCs were first gated for lymphocytes (SSC-A vs. FSC-A), singlet forward scatter dot plots (FSC-W vs. FSC- A), singlet side scatter dot plots (SSC-W vs. SSC- A) and the lymphocyte gate was further analysed for its uptake of the Live/Dead Aqua stain to determine live versus dead cells and their expression of CD3 and CD4, taking only the live, healthy T-cell population. CD4 surface expression was then determined from this gated population that had earlier been optimized to detect T-conventional ($CD4^{+}CD25^{Low}$) cells and T-regulatory ($CD4^{+}CD25^{high}$) cells as appropriate. Expression of the proportion of Tregs in the $CD4^{+}$ lymphocyte gate was considered as an intercept of three gates based on the combination of staining cell surface markers (CD4, CD25, CD127) and intracellular staining of transcription factor forkhead box protein 3 (FoxP3). Histograms show a representative example of the expression of C3aR, C5aR1, C5aR2 (C5L2) and CD46 (numbers represent geometric mean fluorescent intensity), either on the surface or intracellularly of $CD4^{+}CD25^{Low}$ and $CD4^{+}CD25^{high}$ from healthy, low-risk MDS and high-risk MDS samples. A minimum of 10,000 events was collected per sample. Data were analysed using a FACS Canto II or Fortessa Flow Cytometer.

3.3 Results

3.3.1 Optimisation of test protocol

Here I address the question of assessing complement receptor expression levels in $CD4^{+}$ T-cells from PBMC samples taken from three different donor population: healthy donor (HD), low risk MDS (LR-MDS) and high risk MDS (HR-MDS). It was observed that there was no significant change in the intracellular expression of CD46 in Treg and Tconv cells in the presence or absence of anti-CD3 and CD46 at time points of 1 min and 1hr, and between 24 and 48hr. This led to choosing the 24hr time point for subsequent experiments involving LR- and HR-MDS patients, owing to the low number of cells from these donors.

3.3.2 Comparison of intracellular complement receptor expression in healthy vs MDS donors

Following the protocol optimisation and the selection of the 24hr time point the activation of T-cells by the addition of anti-CD46 to PBMC, its ability to regulate intracellular complements expression was compared in healthy vs MDS donors. In all the Figures shown below, the left-hand figures represent the means of the 10 donors in each treatment group while the right-hand figures represent the data for each individual in each treatment group.

3.3.3 Intracellular complement receptors expression in $CD4^{+}CD25^{high}$ T-cells (Tregs)

3.3.3.1 Intracellular complement receptors C5aR2 (C5L2)

When PBMCs were stimulated with anti-CD3 plus anti-CD46 it was observed that Tregs in the HR-MDS group expressed significantly higher levels of C5a receptor 2 (C5aR2, C5L2) **(Figure 3-2)** than the HD (P=0.0042) and LR-MDS (P=0.003) groups. Thus, CD46 induced an upregulated C5L2 expression of Tregs in high-risk MDS patients.

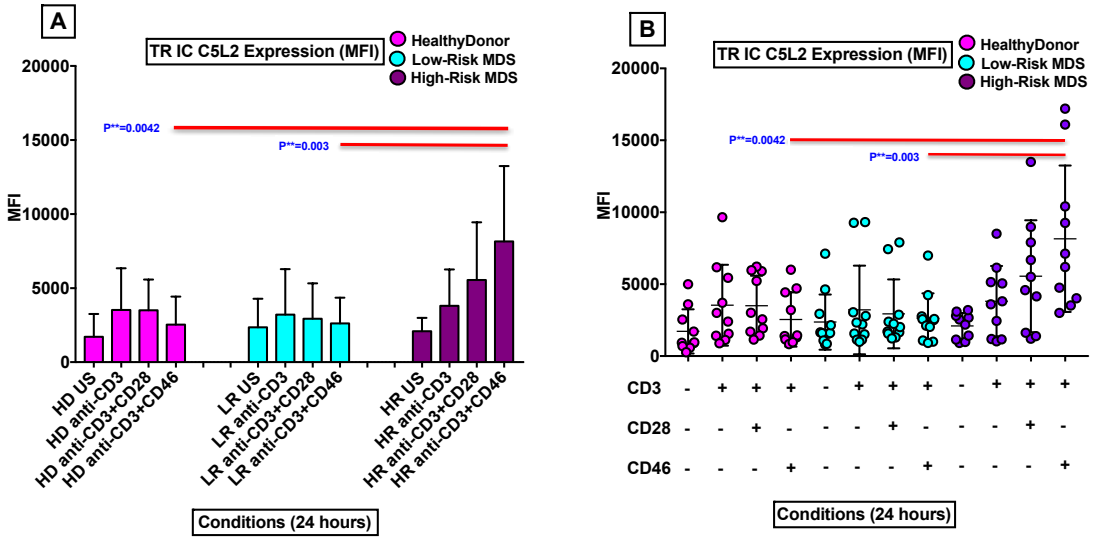


Figure 3-2: CD46 activation on high-risk MDS Treg cells induces intracellular C5L2 up-regulation.
PBMCs cultured in 48-well plates were activated for 24 h with mAbs anti-CD3, anti-CD3±CD28 or anti-CD3±CD46 antibodies. Intracellular C5L2 expression in healthy donor, low-risk-MDS and high-risk MDS Treg cells was measured at different time points by FACS analysis. The data show the mean ± SD derived from healthy donor (n=10), low-risk MDS (n=10) and high-risk MDS (n=10) experiments.

3.3.3.2 Intracellular CD46, C3aR and C5aR1 expression

The data in **Figure 3-S1** show the expression of MCP (CD46) in human Tregs in the three groups (HD, LR- and HR-MDS). Although the intracellular expression of CD46 in both the LR- and HR-MDS patients were lower than in the HD, there was no significant differences between the three-donor population **(Appendix 1).** Although not statistically significant, the expression of both C3aR **(Figure 3-S2)** and C5aR1 **(Figure 3-S3)** were higher in HR-MDS compared to both HD and LR-MDS donors when treated with anti-CD3+CD46 **(Appendix 1).**

3.3.4 Intracellular complement receptor expression in $CD4^{+}CD25^{-/low}$ Tconv cells

3.3.4.1 Intracellular expression of C3aR in human Tconv cells

The expression of intracellular C3aR in T-conv cells in the HR group increased **(Figure 3-3)** compared to the both HD and LR-MDS donors. This increase was statistically significant in the HR-MDS vs HD groups pre- (P=0.0117) and post-stimulation (P=0.0027) with anti-CD3, anti-CD3+CD28 (P=0.0092) or anti-CD3+CD46 (P=0.0113).

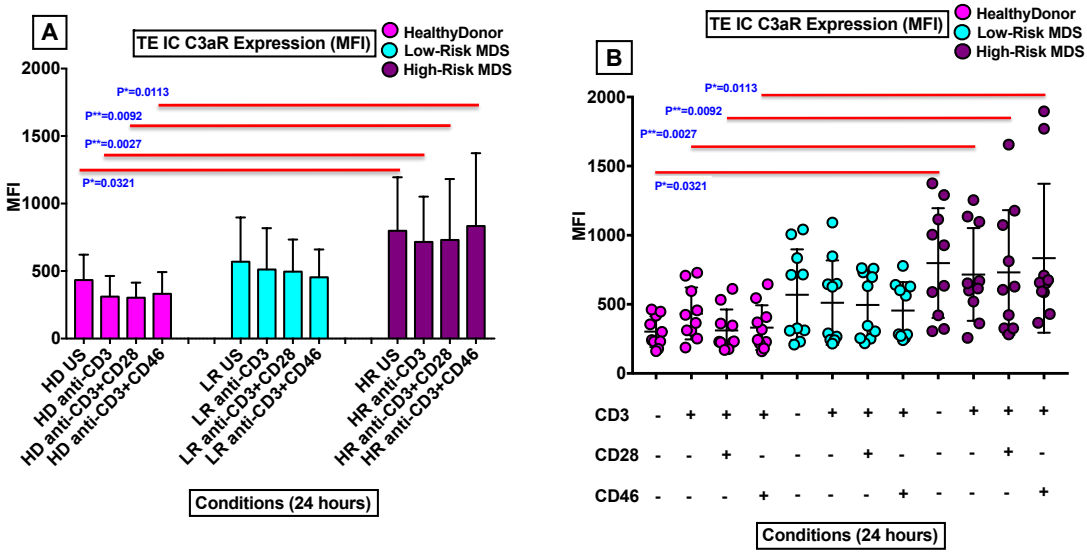


Figure 3-3: CD46 activation on high-risk MDS upregulates the intracellular C3aR expression.
Intracellular C3aR expression of $CD4^{+}CD25^{-}$ T-cells (Tconv) was monitored at 24 h by FACS analysis. Anti-CD3/CD46 activated cells upregulate C3aR in HR-MDS. The data show the mean ± SD derived from HD (n=10), LR-MDS (n=10) and HR-MDS (n=10) experiments.

3.3.4.2 Intracellular expression of C5aR1 in human Tconv cells

$CD4^{+}$ T-cells stimulated with anti-CD3±CD46 antibody induced upregulated C5aR1 expression in $CD4^{+}CD25^{-/low}$ Tconv cells in the HR-MDS group compared to the LR (P=0.0009) and HD groups (P=0.0054) **(Figure 3-4).** Also, a significant difference was detected between the non-activated cells and those stimulated with anti-CD3+CD46 antibody, with C5aR1 being significantly expressed more in the activated cells compared to the non-activated cells (P=0.0032).

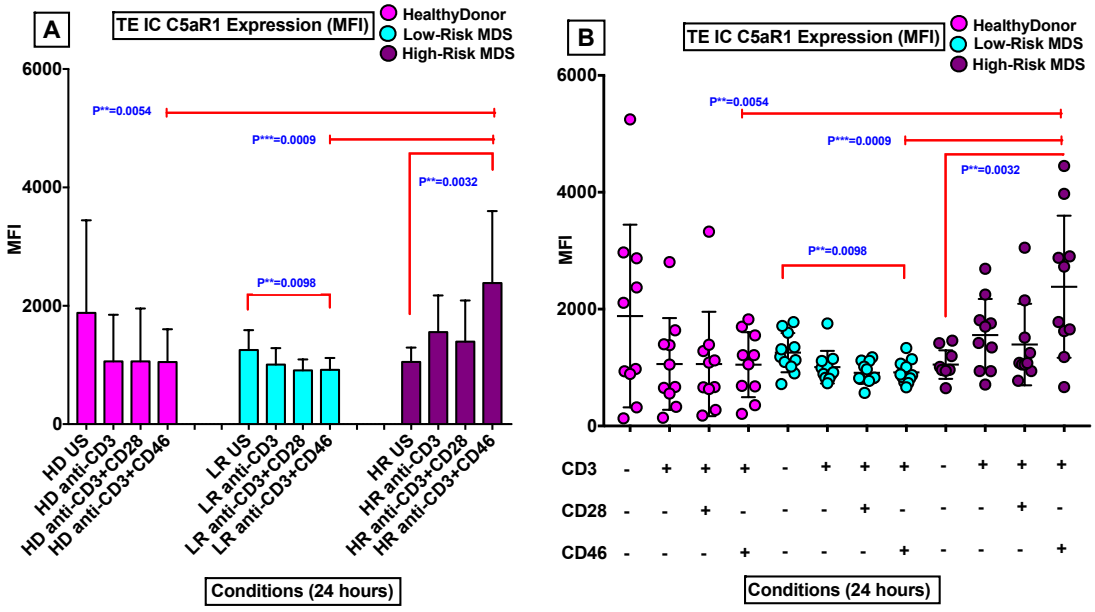


Figure 3-4: Post-CD46 activation of Tconv in high-risk MDS induces overexpression of intracellular C5aR1:

PBMCs were activated for 24 h with an anti-CD3±CD46 antibody. The $CD4^{+}CD25^{-}$ T-cell (Tconv) intracellular C5aR1 expression was measured at the indicated time points. The data show the mean ± SD derived from ten experiments (n=10) for each group.

3.3.4.3 Intracellular CD46 and C5L2 expression in human Tconv cells

Here, no significant difference was found in the intracellular expression of CD46 **(Figure 3-S4)** and C5L2 **(Figure 3-S5)** in Tconv cells between the three groups (HD, LR- and HR-MDS) **(Appendix 1)**.

3.3.5 Surface expression of complement receptors by Tregs

3.3.5.1 Expression of C3aR on the surface of Treg

The results demonstrated low surface expression of C3aR on Treg cells of the HR-MDS donors compared to the both HD and LR-MDS donors **(Figure 3-5)** with significant differences between the HR-MDS and HD samples after stimulation with anti-CD3 ($P<0.0001$), anti-CD3+CD28 ($P=0.0002$) or anti-CD3+CD46 ($P=0.0003$). Also, there was low expression of C3aR in the Treg cells of the LR- group compared to the HD, with significant changes in the LR-MDS and HD groups before activation ($P=0.0027$) and after stimulation with anti-CD3+CD28 ($P=0.0027$) or anti-CD3+CD46 ($P=0.033$).

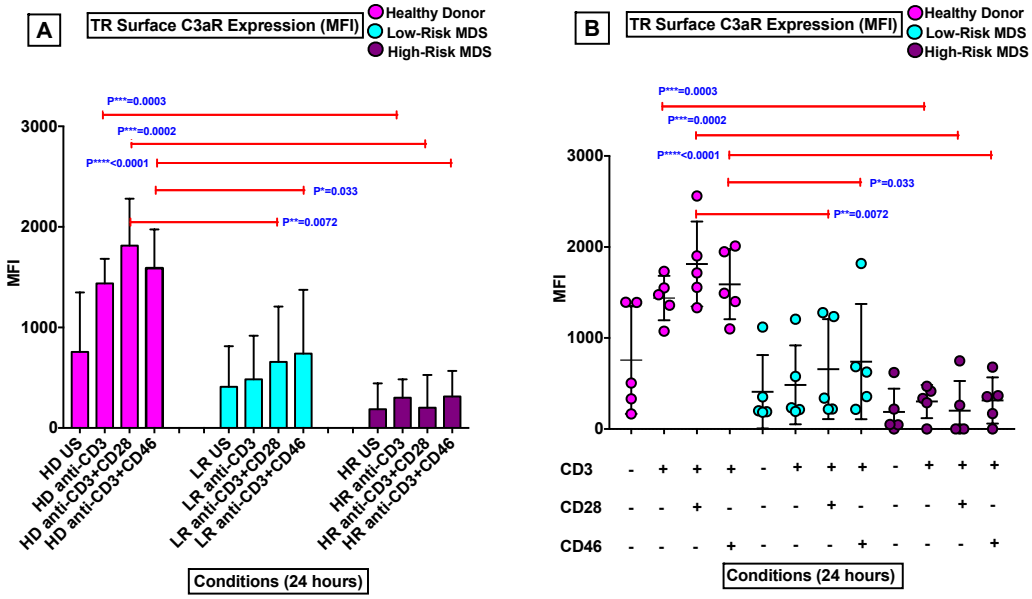


Figure 3-5: Down-regulation of C3aR expressions on the surface of Treg.
Surface C3aR expression on $CD4^{+}CD25^{high}$ T-cells (Treg) was monitored at 24 h by FACS analysis. The results show low levels of C3aR expression on the surface of Tregs in LR- and HR-MDS. The data show the mean ± SD derived from healthy donor (n=5), LR-MDS (n=5) and HR-MDS (n=5) experiments.

3.3.5.2 Expression of C5L2 on the surface of Treg

There was very low surface expression of C5L2 on the surface of non-activated $CD4^{+}CD25^{high}$ Treg cells of LR- and HR-MDS patients compared to healthy donors, (P= 0.0045 and P=0.0022 respectively) **(Figure 3-6).**

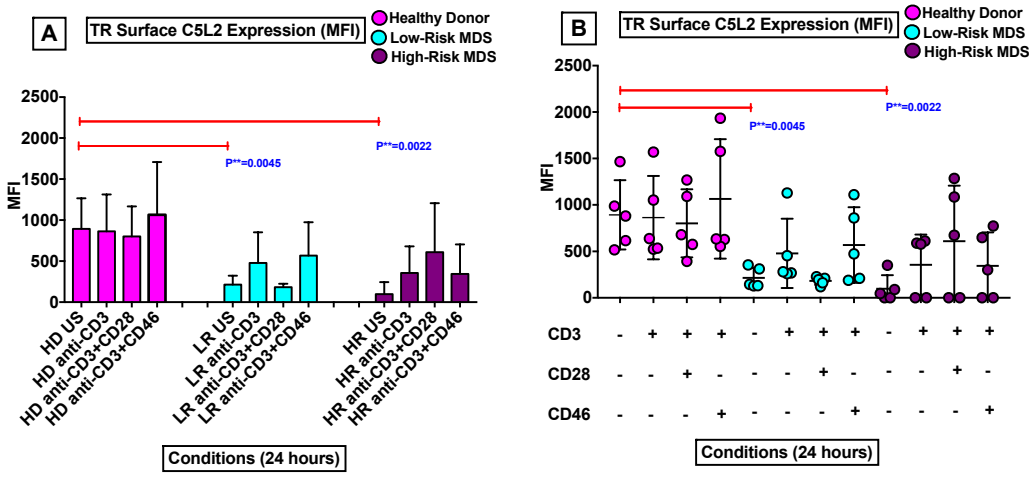


Figure 3-6: Expression of C5L2 on the surface of human Treg cells using FACS analysis.
PBMCs were activated for 24 h with mAbs anti-CD3, anti-CD3±CD28 or anti-CD3±CD46 antibodies. Surface C5aR2 (C5L2) expression of healthy donor, low-risk-MDS and high-risk MDS Treg cells were then measured at the 24 h time point by FACS analysis. The data show the mean ± SD derived from HD (n=5), LR-MDS (n=5) and HR-MDS (n=5) experiments.

3.3.5.3 Expression of CD46 and C5aR1 on the surface of Treg

It was noticed that no significant change in the expression of CD46 **(Figure 3-S6)** and C5aR1 **(Figure 3-S7)** on the surface of $CD4^{+}CD25^{+}$ Treg cells in all three groups, although C5aR1 expression was higher in HD compared to MDS donors **(Appendix 1).**

3.3.6 Surface expression of complement receptors by Tconv

3.3.6.1 Expression of CD46 on the surface of Tconv

Figure 3-7 shows the expression of human MCP (CD46) on the surface of human Tconv cells in all three groups (HD, LR- and HR-MDS). There was high surface expression of CD46 in the HR-MDS patients compared to the HD and LR-MDS groups, and significant differences were found between levels of CD46 in the HR- and LR-MDS groups. Low surface expression of CD46 was recorded on the Tconv cells of the LR-MDS group just after stimulation with anti-CD3 (P=0.042), anti-CD3+CD28 (P=0.0351) or anti-CD3+CD46 (P=0.0359) compared to HR-MDS.

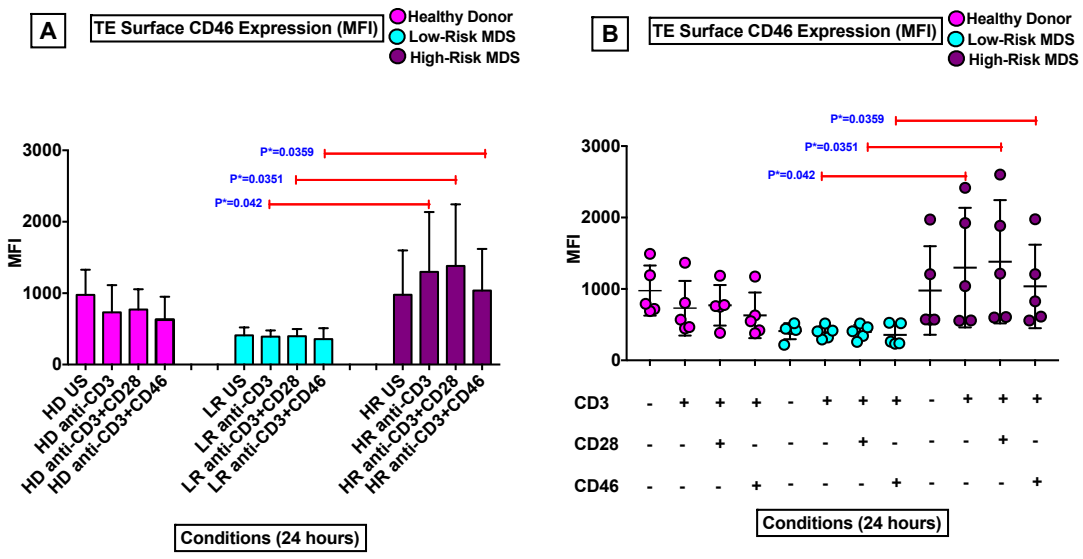


Figure 3-7: MCP (CD46) expressions on the surface of human T-cells (Tconv)
PBMCs were activated for 24 h with an anti-CD3, anti-CD3±CD28 or anti-CD3±CD46 antibodies. Surface CD46 expression of Treg cells was measured at the 24 h time point. Results are representative of fifteen (n=15) experiments and the data show the mean ± SD derived from HD (n=5), LR-MDS (n=5) and HR-MDS (n=5) experiments.

3.3.6.2 Expression of C5aR1 on the surface of Tconv

The data showed low expression of C5aR1 in the Tconv cells of the LR-MDS group **(Figure 3-8).** The expression levels in both HD and HR donors were significantly higher (P=0.0009, 0.027 respectively) than the LR donors when cells were stimulated with anti-CD3+CD46. On the other hand, no significant difference was noted in the surface expression of C5aR1 between the Tconv cells of HR-MDS and those of the HD.

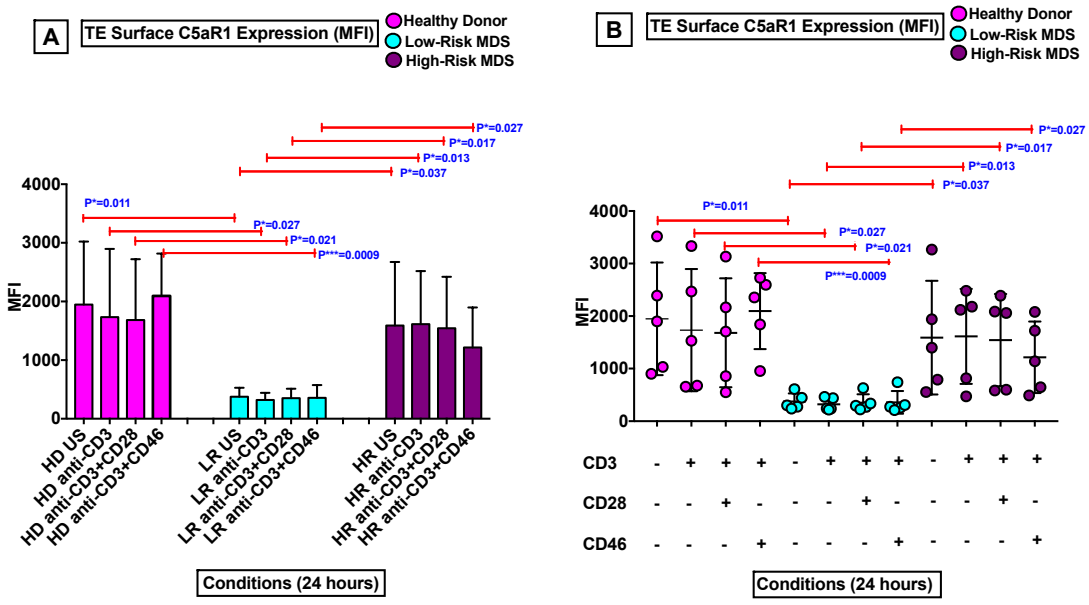


Figure 3-8: C5aR1 expression on the surface of (Tconv).
PBMCs were activated for 24 h with an anti-CD3±CD46 antibody. C5aR1 expression on the surface of Tconv cells was measured at the 24 h time point. The data show the mean ± SD derived from ten experiments (n=5) for each group.

3.3.6.3 Expression of C5L2 on the surface of Tconv

C5L2 expression on the surface of T-conventional cells of HD-MDS patients before and after stimulation with anti-CD3 (P=0.026), anti-CD3+CD28 (P=0.048) or anti-CD3+CD46 (P=0.031) was markedly higher than that for the LR-MDS group **(Figure 3-9).** Very low expression was noticed on the surface of T-conv cells of LR-MDS patients before stimulation (P=0.0013), and after stimulation with anti-CD3 (P=0.0079), anti-CD3+CD28 (P=0.0057) or

anti-CD3+CD46 (P=0.025) compared to the HD group **(Figure 3-9).** The expression levels were not significant between the HR-MDS and HD groups.

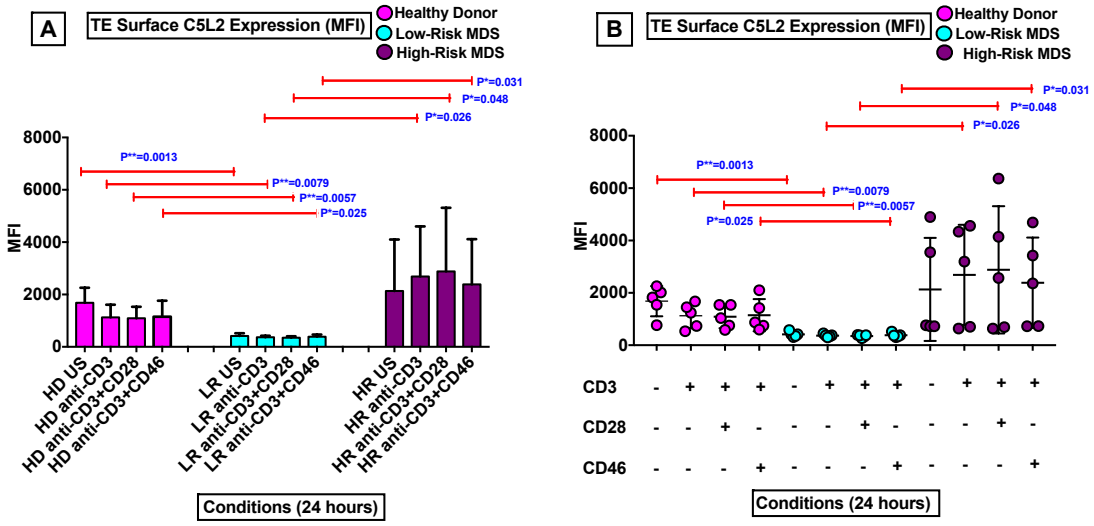


Figure 3-9: C5L2 expression on the surface of Tconv cells
T-cells were cultured in 48-well plates and then activated with mAbs to anti- CD3±CD46 antibody. C5L2 expression was measured on the surface of Tconv at the time points shown by FACS analysis. The data show the mean ± SD derived from five experiments (n=5) for each group.

3.3.6.4 Expression of C3aR on the surface of Tconv

There was no significant difference in the surface expression of C3aR on Tconv cells between the three groups **(Figure 3-S8),** although the expression was higher in the HD **(Appendix 1).**

Table 3-1: Summary of the results of intracellular and surface staining of complement receptors in Tregs and Tconv cells

Complement receptor	Intracellular staining		Surface staining	
	Treg	Tconv	Tregs	Tcon
CD46	↑HD than LR & HR	→ HD, LR & HR	↑HD than LR & HR	↑↑HR than LR
C3aR	↑HR than LR & HD	↑↑HR than LR & HD	↑↑HD than LR & HR	↑HR than LR & HD
C5aR1			↑HD than LR & HR	↑↑HR & HD than LR
C5L2	↑↑HR than LR & HD	→ HD, LR & HR	↑HD than LR & HR	↑↑HR & HD than LR

↑ is high, ↑↑ is significantly higher; → is similar levels; HD: healthy donors; HR: high risk MDS; LR: low risk MDS.

3.4 $CD4^+$ T-cell expression of complement component C3a+C5a by Western Blot

To determine the expression of complement components (C5a and C3a), Western blotting was conducted. It was observed that in HD **(two donor samples)** the stimulation of $CD4^+$ T-cells with anti-CD3+CD46 resulted in downregulation of both C5a **(Figure 3-10)** and C3a **(Figure 3-11)** compared to non-activated or anti-CD3+CD28 stimulation. In both HR and LR-MDS donors **(two samples for each)** there was no expression of these two complement components in either the activated or non-activated state. The numbers below the Western blots represented the band intensity (quantification) for the healthy donors **(Figure 3-10 and 3-11).** For MDS samples, quantification could not be performed because there were no detectable bands. Image Studio Lite software was used for normalisation of band intensity.

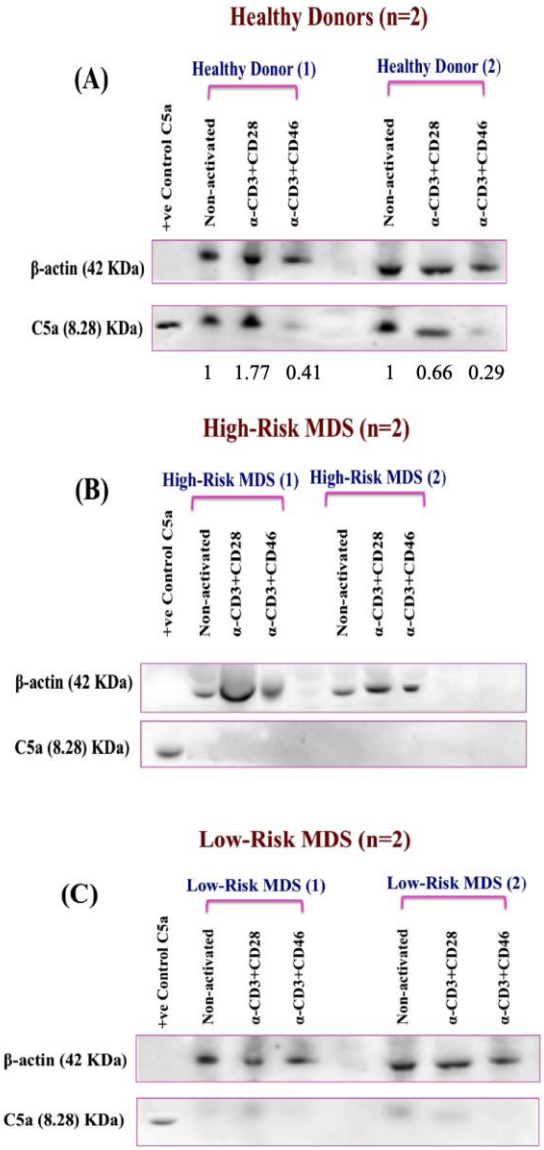


Figure 3-10: Western Blot analysis for Complement Component (C5a). Western blot analyses of cell lysates from 24h activated $CD4^+$ T-cells assessed for C5a (8.27-kDa) expression. Cells were left non-activated or activated with anti-CD3+CD28 or anti-CD3+CD46. β-actin levels (42-KDa) were measured as loading control. Precision plus protein dual extra prestained protein standards as a loading marker (2-250 KDa). Anti-human complement component C5a antibodies used as a positive control to only recognize a C5a molecular weight. Data in Figures A-C are representative of 2 independently performed experiments using a different donor each time A) Healthy donor B) HR-MDS and C) LR-MDS.

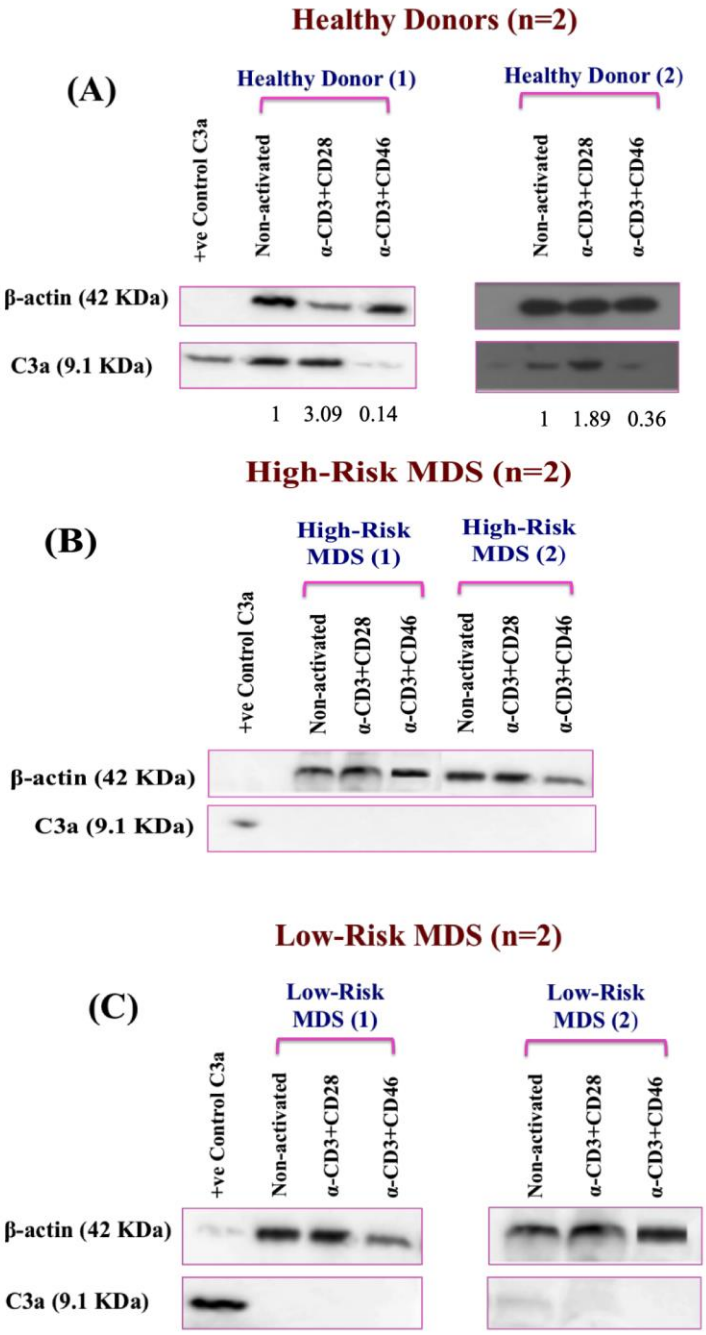


Figure 3-11: Western Blot analysis of C3a expression in CD4$^+$ T-cells. Western blot analyses of cell lysates from 24h activated CD4$^+$ T-cells assessed for C3a (9.1-kDa) expression. Cells were left non-activated or activated with anti-CD3+CD28 or anti-CD3+CD46. β-actin levels (42-KDa) were measured as loading control. Precision plus protein dual xtra prestained protein standards as a loading marker (2-250 KDa). Anti-human complement component C3a antibodies used as a positive control to only recognize a C3a molecular weight. Data in Figures A-C are representative of 2 independently performed experiments using a different donor each time A) Healthy donor B) HR-MDS and C) LR-MDS.

3.5 Discussion

In this study I compared the expression of intracellular and surface complement receptors (CD46, C3aR, C5aR1, and C5L2) in Treg and Tconv $CD4^+$ cells in healthy versus low-risk and high-risk MDS patients. Additionally, complement component C3a and C5a expression, both of which are anaphylatoxins were determined. To do these, I initially optimised a protocol to determine the optimum time point for effective expression of these markers. I observed that culturing PBMC from healthy donors for a period of 24hr was ideal for the test protocol.

For intracellular staining of complement receptors, I observed a significantly higher expression of C5L2 after stimulation with anti-CD3+CD46 in Tregs of HR-MDS donors than healthy and LR-MDS donors, and a significantly higher expression of both C3aR and C5aR1 in Tconv cells of HR-MSD than in healthy and LR-MDS donors. In contrast to the intracellular staining observation above, surface C5L2 was down-regulated on the surface of inactivated $CD4^+CD25^+$ Tregs cells of LR and HR-MDS patients compared to healthy donors. On the other hand, the expression of C5L2 on the surface of Tconv cells of HR-MDS samples before and after stimulation with anti-CD3, anti-CD3+CD28 or anti-CD3+CD46 was markedly higher than in LR-MDS donors.

Defects in $CD4^+CD25^{high}$ Tregs have been observed in different autoimmune diseases (Astier et al., 2006). Complement signalling through C3aR and C5aR have been shown to inhibit Tregs expression and function through a C3a/C5a-induced phosphorylation of AKT phosphorylate (Le Friec et al., 2013). Other studies have also demonstrated that complement receptor signalling via interaction of $CD4^+$ T-cells and dendritic cells up-regulates co-stimulatory molecule expression, thus enhancing TH1 and TH17 T effector function and increasing the survival of Tconv cells (Strainic et al., 2013). Intracellular complement components are one of the key players in initiating and regulating T-conventional and T-regulatory T-cell responses (Heeger and Kemper, 2012). The higher C5L2 expression in HR-MDS donors in Tregs may result in inhibition of C3a and C5a and thereby making C3aR and

C5aR1 available for binding by Tconv cells. This result was confirmed by the output from a Western blot in which both HR-MDS and LR-MDS donors showed no expression of C3a and C5a complement components in both activated and non-activated states. Thus, C5a and C5aR signalling in $CD4^+$ T-cells potentiate immune responses, while the absence of this signalling could convert naïve T-cells to an iTreg response (Strainic et al., 2013). Alternatively, it is possible that patients with MDS might have some defect in $CD4^+$ T-cells and that might have accounted for their inability to express the two complement components (C3a and C5a).

My findings have shown significantly high surface expression of CD46 in Tconv of HR-MDS patients compared to the HD and LR-MDS patients. This observation was so in both pre- and post-stimulation with anti-CD3, anti-CD3+CD28 or anti-CD3+CD46.

With respect to surface staining, significant expression levels of complement receptor C3aR were observed in Tregs cells of HR-MDS patients compared to HD and LR-MDS patients. On the contrary, in Tconv cells, significant expression levels of three of the four complement components CD46, C5aR1 and C5L2 were observed in HR-MDS patients compared to HD and LR-MDS patients. The expression of C3aR is inducible under inflammatory conditions both *in vitro* and *in vivo* with T-cells from patients with signs of severe systemic inflammatory reactions noticeably expressing C3aR (Werfel et al., 2000). Additionally, the reduction of intracellular T-cell C3aR expression induces a decrease in mTOR activity and cell viability similar to that induced by the CSTL inhibitor, implying that intracellular C3a generation and C3aR ligation contribute to mTOR activity and overall T-cell survival (Clarke and Tenner, 2014). Recently, some studies have reported the role of C5L2 in regulating inflammation (Chen et al., 2007). In addition, C5L2 has also been shown to contribute to signalling triggered by C5a or C3a with C5aR or C3aR (Chen et al., 2007). Intriguingly, it has been found that C5L2 may be critical in upregulating cell surface expression of C5aR, particularly in T-conv cells, which suggests that C5L2 is capable of transducing signals; this may be how C5L2 contributes to C5a signal optimization. The absence of signalling via C3aR and C5aR resulted in the lower expression of co-stimulatory molecules and interleukin 6 (IL-6) and more

production of IL-10. The resulting iTreg cells exerted robust suppression, showed enhanced stability, and suppressed ongoing autoimmune disease. Antagonism of C3aR and C5aR can also induce functional human iTreg cells (Strainic et al., 2013). Additional studies exhibited that signalling through C5L2, and not C5aR, leads to inflammatory protein release and thus the proinflammatory role of C5L2 is posited (Bamberg et al., 2010). C5L2 becomes activated only after C5aR and ligand binding, thus functioning as an intracellular receptor.

3.6 Perspective

Collectively, in this study I observed no expression of C3a and C5a, subsequent lack of signalling through C3aR and C5aR along with upregulation of C5aR2 (C5L2), an alternative receptor for C5a, in MDS patients suggesting a potential mechanism for Treg expansion in MDS. Moreover, the contribution of C5L2 in regulating pro-inflammatory and anti-inflammatory responses and, in particular, certain features of the complement signalling pathway, makes it a possible agent for remedying diseases such as MDS. The next chapter will examine levels of complement associated mTOR and TGF-β signalling pathway-associated proteins in healthy donors and patients with LR- and HR-MDS.

Chapter 4. Results II

Levels of mTOR, TGF-β Signalling Pathway-Associated Proteins, and Cytokines in Patients with MDS

4.1 Introduction

Intracellular complements (particularly C3a and C5a) play a crucial role in the polarization of $CD4^+$ T-cells toward regulatory or effector phenotypes through the TGF-β pathway (C5aR2 mediated) or mTOR (C5aR1 mediated) respectively. However, the C5L2 expression and its relationship with Treg suppressor function and stability are not known. To address the current knowledge gap, I sought to explore whether the stimulation of complement receptors (C3aR, C5aR) with exogenous C3a and C5a may lead to the hyper-activation of the mTOR pathway instead of the SMAD signalling pathway and any potential increases in the number of Th1 cells. The aim of this present chapter is to investigate the potential role of mTOR and Akt as important proteins in complement-related polarization of $CD4^+$ T-cells towards pro-inflammatory T helper cells MDS. The TGF-β signalling pathway and its related proteins were also studied which are activated following stimulation with anti-CD46 antibody in $CD4^+$ T-cells from healthy donor (HD), LR and HR-MDS patients. Furthermore, responses following treatment with C3a and C5a were examined. Also, the supernatant concentrations of cytokine and chemokine were evaluated in MDS patients.

4.2 Results

4.2.1 The role of complements components in levels of mTOR and TGF- β signalling Pathway-Associated Proteins in Patients with MDS

TGF-β signalling pathway proteins pSMAD2, pSMAD3 and pSMAD4 as well as mTORc were evaluated in human $CD4^+$ T-cells of 6 healthy donors and 12 MDS patients (out of which 6 had LR and 6 had HR-MDS).

4.2.1.1 mTOR

Regarding mTOR signalling pathway, CD46 induced a significant down-regulation of pmTOR levels in HR-MDS patients. Before the treatment with C3a+C5a, the levels of pmTOR decrease in the HR-MDS (P=0.0278) as compared to healthy donors. While, after treatment with C3a and C5a, the levels of pmTOR in the HR-MDS (P=0.0441) increased slightly but were still not to the level as in the HD. Although the level of pmTOR is downregulated in LR-MDS after stimulation with CD46, the data show no significant decrease in protein levels of mTOR as compared to HD **(Figure 4-1)**.

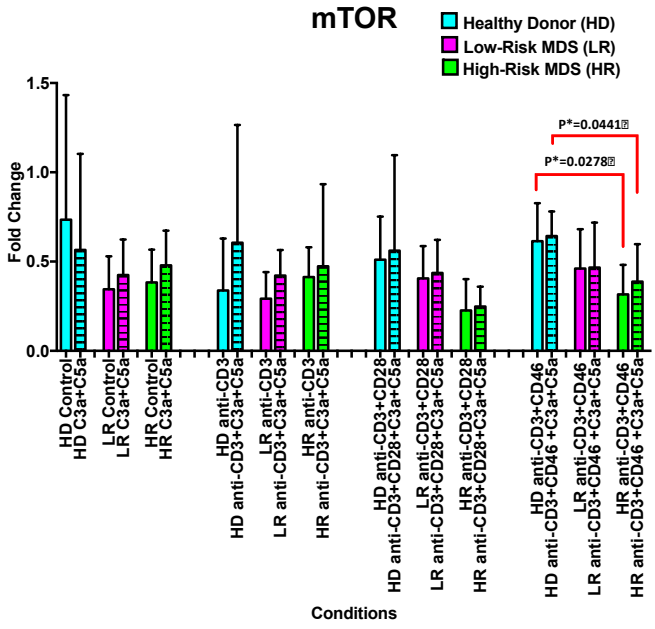


Figure 4-1: mTOR expression following $CD4^+$ T-cell stimulation and C3a+C5a treatment.
$CD4^+$ T-cells, from healthy (n=6), LR-MDS (n=6) and HR-MDS donors (n=6) without (−) or with (+) stimulation (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of 50nM C3a and C5a of each. Error bars indicate SD of six experiments. Data are representative of six experiments with similar results.

4.2.1.2 pSMAD 2, 3 & 4

Following stimulation with anti-CD3±CD46 for 24 hours, $CD4^+$ in HR-MDS patients showed a significantly lower level of pSMAD2 (p=0.016) **(Figure 4-2),** pSMAD3 (p=0.0110) **(Figure 4-3)** and pSMAD4 (p=0.0017) **(Figure 4-4)** in comparison to HD. However, in pSmad2 following CD46 stimulation, the combination of C5a and C3a induced an increase in pSmad2 levels (p=0.0442) in HR-MDS compared to HD **(Figure 4-2).** In HR-MDS patients the level of pSMAD4 increased after 24 hours of stimulation with anti-CD3 and CD46 plus C3a and C5a (p=0.027) **(Figure 4-4)** compared to HD. Moreover, in HR-MDS patients the level of pSMAD3 (p=0.0258) remained significantly lower than LR-MDS after 24 hours of stimulation with anti-CD3 and CD46 plus C3a and C5a **(Figure 4-3)**. However, no noticeable changes were noted in the protein levels of p-SMAD3 **(Figure 4-3)** and p-SMAD4 **(Figure 4-4)** were seen between the HD and LR-MDS. In p-SMAD4 there was a significant difference between the HR and LR-MDS patients (p=0.0005) when activated with anti-CD3+CD46.

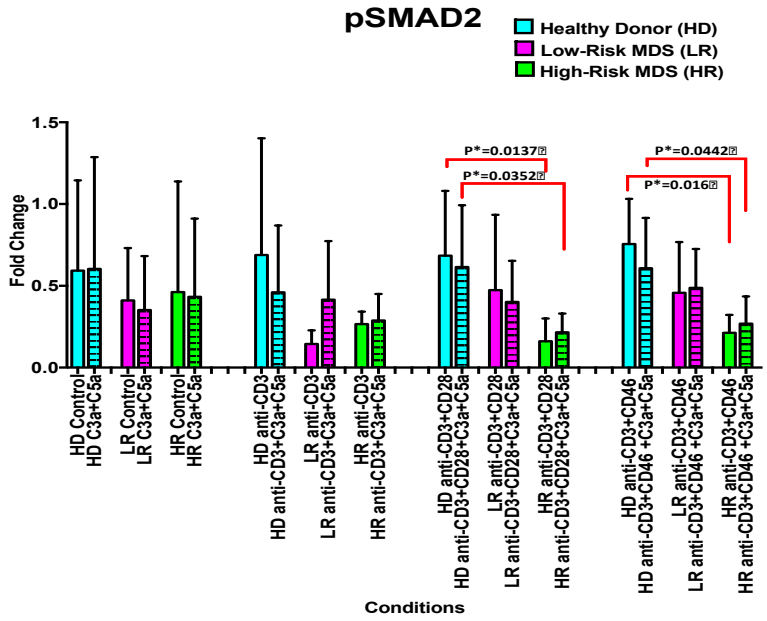


Figure 4-2: pSMAD2 signalling in HD, LR and HR-MDS
Naïve $CD4^+$ T-cells isolated from PBMCs of healthy (n=6), LR-MDS (n=6) and HR-MDS (n=6) donors without (−) or with (+) stimulation (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of C3a 50nM and C5a 50nM. Error bars indicate SD of six experiments. Data are representative of six experiments with similar results.

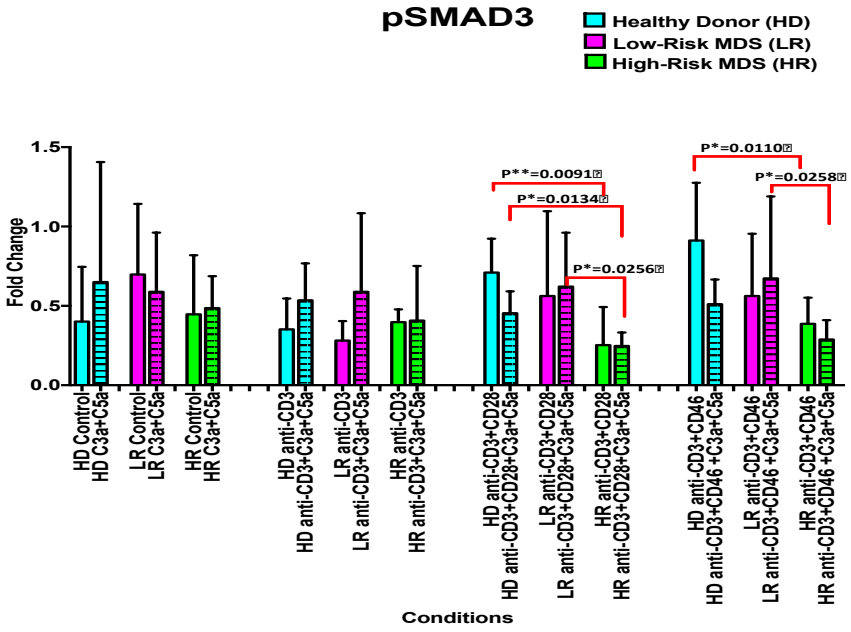


Figure 4-3: p-SMAD3 protein levels
CD4^{+} T-cells from healthy, LR-MDS and HR-MDS donors were left inactivated or activated for 24 h with mAbs anti-CD3, anti-CD3+CD28 or anti-CD3+CD46 antibodies and cells were untreated or treated with 50nM C3a and 50nM C5a. The data show the mean ± SD derived from HD (n=6), LR-MDS (n=6) and HD MDS (n=6) experiments. Data are representative of six experiments with similar results.

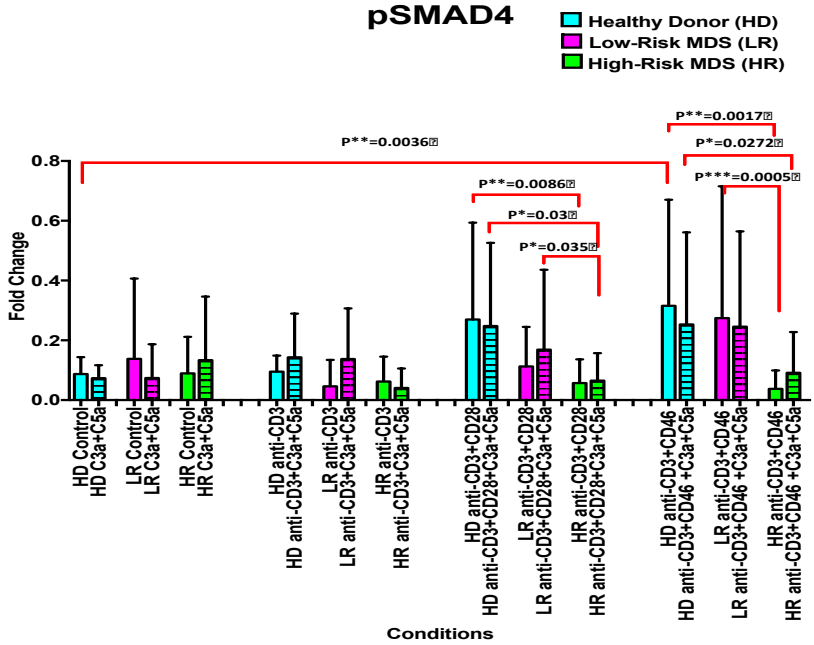


Figure 4-4: Defect in pSMAD4 signalling in HR-MDS after stimulation.
CD46 without C3a+C5a downregulated pSMAD4 and CD46 plus C3a+C5a up-regulated it in naïve CD4^{+} of HR-MDS cells compared to healthy and LR-MDS cells. Error bars indicate SD of six experiments. Data are representative of six experiments with similar results.

4.2.1.3 pAKt, pERK1/2 and TGFbRII

In pAKt, independent of the culture condition, there was no significant difference in fold change between HD, HR and LR-MDS **(Figure 4-S1) Appendix 2.**

The protein level of pERK1/2 showed decrease in LR-MDS compared to HD and HR-MDS**.** PERK1/2 was down-regulated by CD3+CD46 alone and up-regulated in the presence of C3a and C5a except in LR-MDS which had no response after C5a and C3a treatment. However, no noticeable changes in the protein levels of p-ERK1/2 were found between HD and HR-MDS patients **(Figure 4-S2) Appendix 2.**

Finally, the protein levels of TGFbRII showed no significant changes between HD, LR and HR-MDS before and after complement treatment **(Figure 4-S3) Appendix 2.**

4.2.2 Cytokine secretion in response to stimulation to complement pathway stimulation

The aim of this part of study was to evaluate the cytokine secretion by $CD4^+$ T-cells from MDS patients in response to complement pathway activation. Supernatants from the culture conditions were used for the cytokine assays. It was observed that IL-17A secretion induced by anti-CD3, anti-CD3+CD28, anti-CD3+CD46 co-activation in $CD4^+$ T-cells was increased significantly in LR-MDS patient (from about 1.0 to 1.5 pg/cells $\times 10^3$) compared with HR-MDS (P=0.00660) after complement treatment, and both before and after complement activation **(Figure 4-5)** compared to HD (P=0.0088, 0.0202 respectively) **(Table 4-1).**

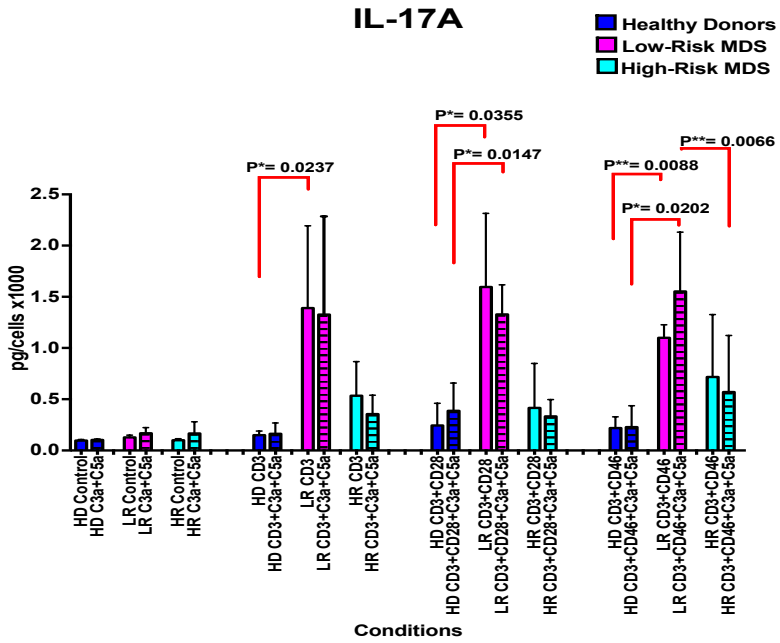


Figure 4-5: IL-17A secretion by $CD4^+$ T-cells in LR-MDS, HR-MDS and HD.

Isolated naïve $CD4^+$ T-cells from PBMCs of healthy (n=3), LR-MDS (n=3) and HR-MDS (n=3) donors were maintained non-activated or activated with CD3 alone, (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of 50nM C3a and C5a for 24h for each. Then the supernatants were collected, and the concentrations of secreted cytokines were measured. Error bars indicate the SD of three experiments.

Table 4-1: Summary of the results of cytokines (IL-17A, IFN-γ, IL-6 and IL-2) (P-value) in HD, LR and HR MD. Red boxes (increased) values.

Conditions		IL-17 A		IFN-γ		IL-2		IL-6		
		LR to HD	LR to HR	LR to HD	LR to HR	LR to HD	LR to HR	LR to HD	LR to HR	HD to HR
Unstimulated	No Treatment					0.0019				
	Treatment (C3a+C5a)									
Anti-CD3	No Treatment	0.0237		0.0253	0.047	0.0015				0.0428
	Treatment (C3a+C5a)									
Anti-CD+CD28	No Treatment	0.0355		0.0029	0.0036	<0.0001				
	Treatment (C3a+C5a)	0.0147		0.0149	0.0173	0.0009		0.0352		
Anti-CD+CD46	No Treatment	0.0088		0.0035	0.0129	0.0022			0.0153	0.0008
	Treatment (C3a+C5a)	0.0202	0.0066	0.0029	0.005	0.0405				

Amongst the pro-inflammatory cytokines secreted by activated $CD4^+$ T-cells, it was observed that IFN-γ was significantly over produced in all the conditions of activation in LR-MDS compared to HR-MDS and HD **(Table 4-1).** IFN-γ concentrations induced by anti-CD3+CD46 co-activation was significantly over secreted in patients with LR-MDS compared to HR-MDS (P=0.005) and HD (P=0.0029) **(Figure 4-6).** IFN-γ secretion rates were 10-20 times over-produced in the LR-MDS patients compared to HD and HR-MDS patient groups. The IFN-γ secretion levels were slightly higher in HR-MDS patients compared to HD, but the difference did not reach statistical significance by activated $CD4^+$ T-cells. The level of IFN-γ secretion in LR-MDS did not change after CD3+CD46 co-activation plus C3a+C5a treatment **(Figure 4-6).**

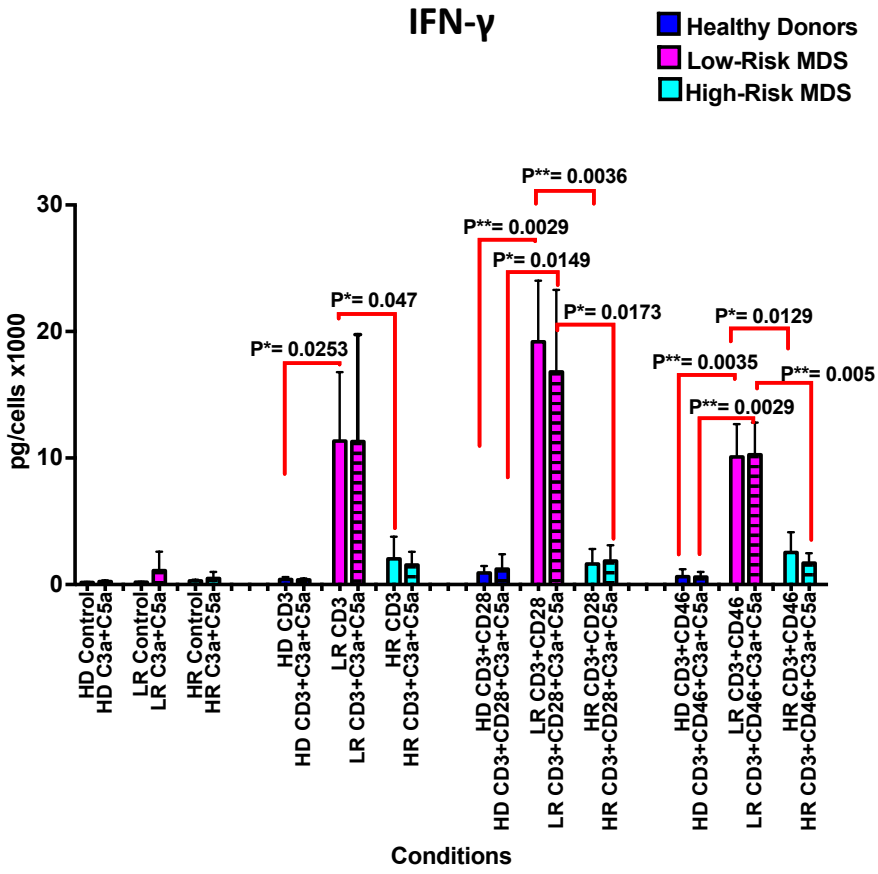


Figure 4-6: IFN-γ secretion by $CD4^+$ T-cells in LR-MDS, HR-MDS and HD.
Isolated naïve $CD4^+$ T-cells from PBMCs of healthy (n=3), low-risk MDS (n=3) and high-risk MDS (n=3) donors were maintained non-activated or activated with CD3 alone, (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) 50nM C3a and 50nM C5a for 24 h. Then the supernatants were collected, and the concentrations of secreted cytokines were measured. Error bars indicate SD of three experiments. Data are representative of three experiments with similar results.

There was a significant tendency for IL-2 to be over-produced (from about 40 to 60 pg/cells $\times 10^3$) in $CD4^+$ T-cells from LR-MDS patients compared to HD, before and after CD3, CD3+CD28 and CD3+CD46 co-activation **(Table 4-1).** The levels of IL-2 were slightly decreased in cells from HR and LR-MDS after anti-CD3+CD46 co-activation plus complement treatment **(Figure 4-7).**

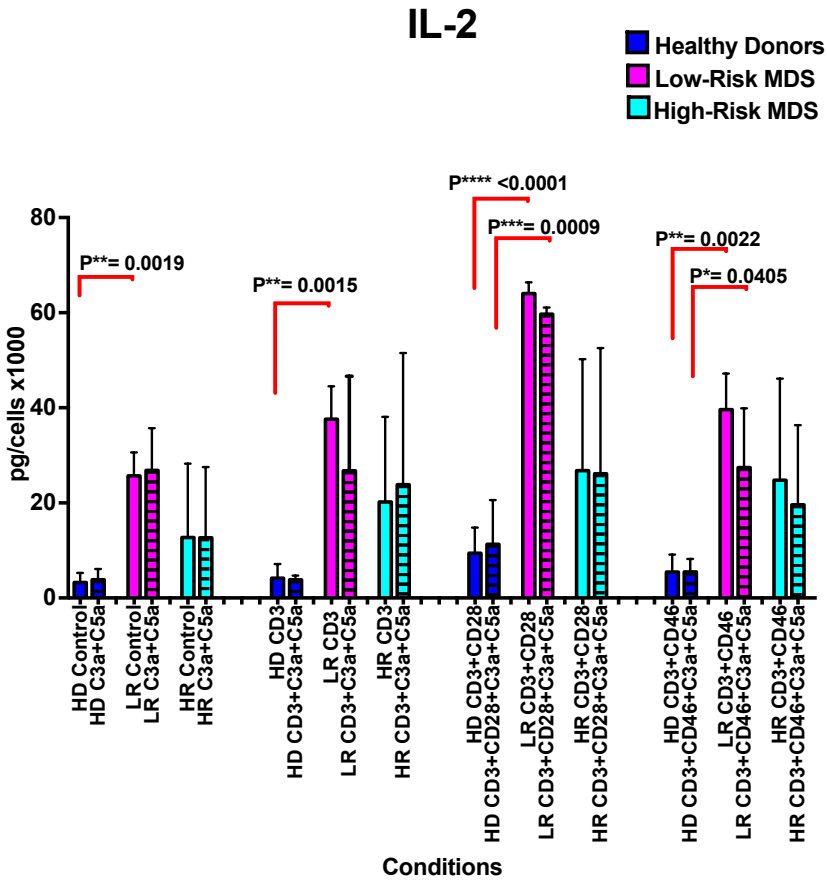


Figure 4-7: Isolated $CD4^+$ T-cells IL-2 secretion profiles
Isolated naïve $CD4^+$ T-cells from PBMCs of healthy (n=3), LR-MDS (n=3) and HR-MDS donors (n=3) were maintained non-activated or activated with CD3 alone, (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of C3a (50nM) and C5a (50nM) for 24 h. Then the supernatants were collected, and the concentrations of secreted cytokines were measured. Error bars indicate SD of three experiments. Data are representative of three experiments with similar results.

Figure (4-8), demonstrate that $CD4^+$ T-cells from patients with LR and HR-MDS have higher IL-6 concentration rates than HD, both when non-activated and also after CD3, CD3+CD28 **(Table 4-1).** After CD3+CD46 stimulation the level of secretion of IL-6 was significantly increased in HR-MDS patents compared to LR-MDS (P=0.0153) and HD (P=0.0008).

Also, it was noted that there was a decrease in IL-6 levels from $CD4^+$ T-cells in patients with both subclasses of MDS after a combination of C3a+C5a treatment, compared to HD.

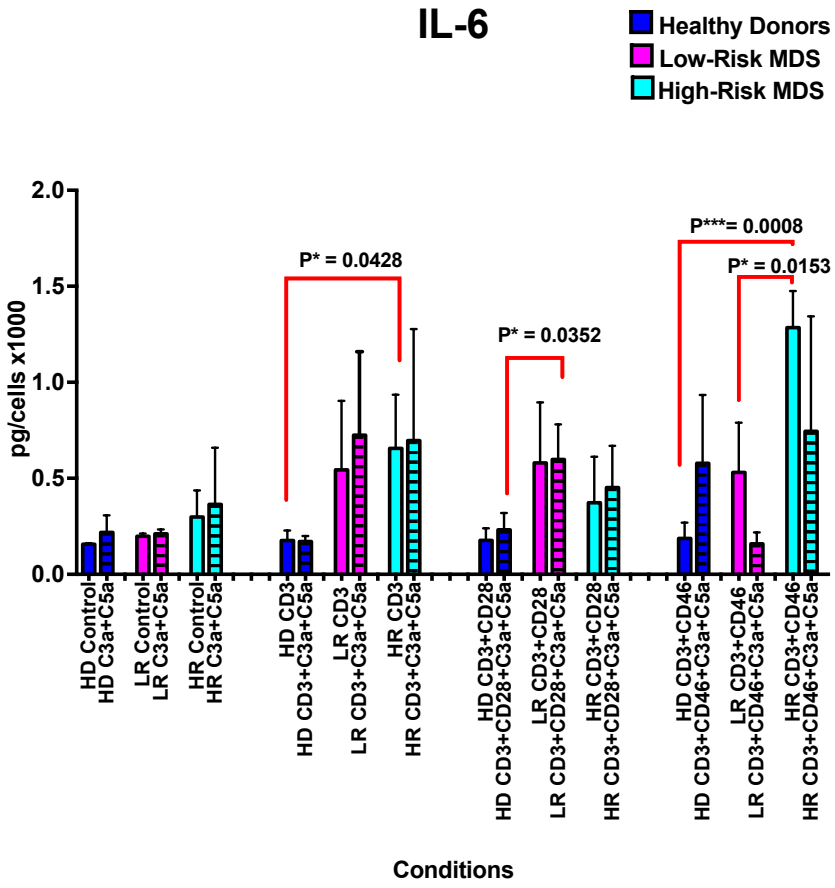


Figure 4-8: $CD4^+$ T-cells IL-6 secretion in LR-MDS, HR-MDS patients and HD is altered by complement treatment.
Isolated naïve $CD4^+$ T-cells from PBMCs of healthy (n=3), LR-MDS (n=3) and HR-MDS (n=3) donors were maintained non-activated or activated with CD3 alone, (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of 50 nM C3a and C5a (for each) for 24 h. Then the supernatants were collected and the concentrations of secreted cytokines were measured. Error bars indicate SD of three experiments. Data are representative of three experiments with similar results.

It was noticed that there was a significant tendency for IL-18 to be over-produced (3-6 folds) in $CD4^+$ T-cells from LR-MDS patients compared to HD and HR-MDS, after anti-CD3, anti-CD3+CD28 and anti-CD3+CD46 co-activation **(Table 4-2).** The levels of IL-18 were slightly decreased in cells from LR-MDS after complement treatment **(Figure 4-9).**

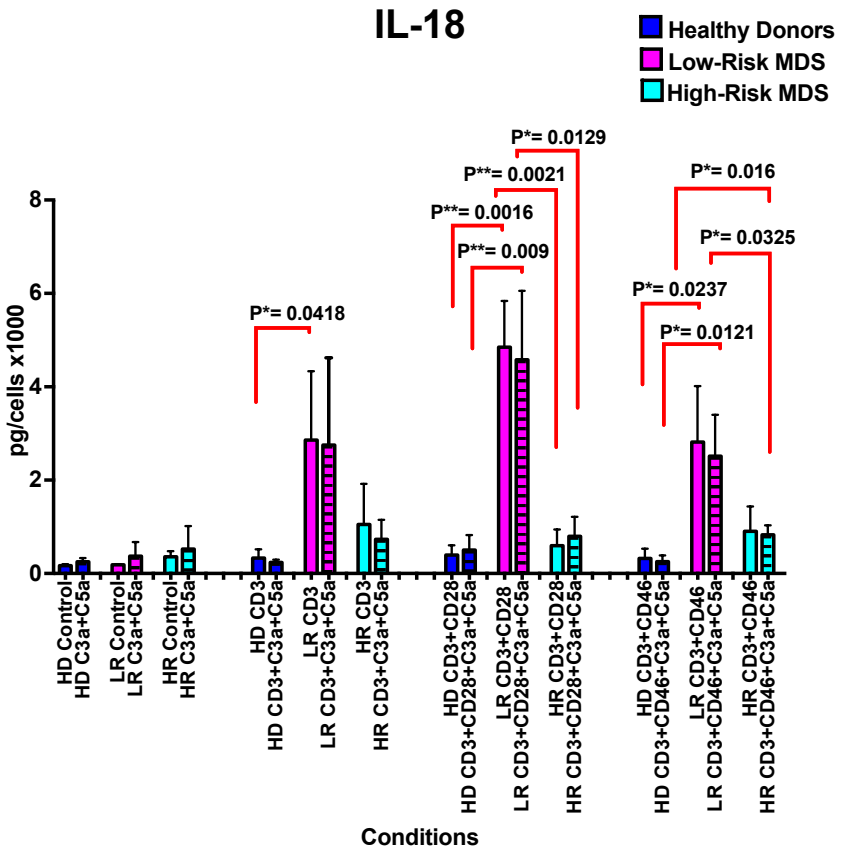


Figure 4-9: IL-18 secretion by CD4$^+$ T-cells in LR-MDS, HR-MDS and HD.
Isolated naïve CD4$^+$ T-cells from PBMCs of healthy (n=3), LR-MDS (n=3) and HR-MDS (n=3) donors were maintained non-activated or activated with CD3 alone, (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of 50 nM C3a and C5a (For each) for 24 h. Then the supernatants were collected and the concentrations of secreted cytokines were measured. Error bars indicate SD of three experiments.

Table 4-2: Summary of the results of cytokines (IL-18, IL-1RA, TNF-α and IL-10) (P-value) in HD, LR and HR-MDS. Red (increased) and green (decreased) values

Conditions		IL-18		IL-1RA		TNF-α		IL-10		
		LR to HD	LR to HR	HR to HD	LR to HR	LR to HD	LR to HR	LR to HD	LR to HR	HD to HR
Unstimulated	No Treatment									
	Treatment (C3a+C5a)									
Anti-CD3	No Treatment	0.0418				0.0348		0.0002	0.0069	
	Treatment (C3a+C5a)			0.0287	0.036			0.0095	0.031	
Anti-CD+CD28	No Treatment	0.0016	0.0021			0.0018	0.0052	0.0024	0.0033	
	Treatment (C3a+C5a)	0.009	0.0129	0.0093	0.0112	0.008	0.02	0.0283	0.03	
Anti-CD+CD46	No Treatment	0.0237		0.0235	0.0262	0.0417		0.0274		0.026
	Treatment (C3a+C5a)	0.0121	0.0325	0.0209	0.023	0.0137		0.0426		0.015

In all the conditions of activation, IL-1RA secretion was a significant higher (15-20 times) in HR-MDS patients compared to LR-MDS and HD **(Table 4-2).** The level of IL-1RA concentration was slightly increased in cells from the HR-MDS group after the combination of C3a+C5a treatment **(Figure 4-10).**

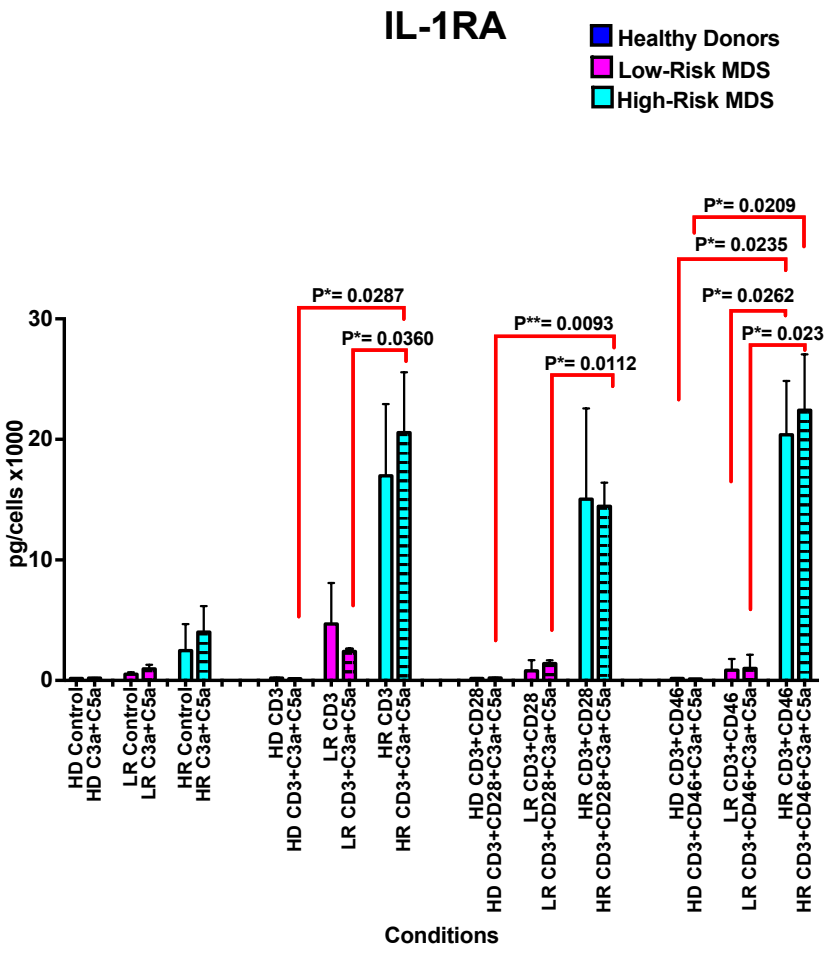


Figure 4-10: Isolated CD4$^+$ T-cells IL-1RA secretion higher in HR-MDS patients.
Isolated naïve CD4$^+$ T-cells from PBMCs of healthy (n=3), LR-MDS (n=3) and HR-MDS (n=3) donors were maintained non-activated or activated with CD3 alone, (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of 50 nM C3a and C5a (for each) for 24 h. Then the supernatants were collected, and the concentrations of secreted cytokines were measured. Error bars indicate SD of three experiments. Data are representative of three experiments with similar results.

TNF-α secretion patterns were analysed and it was found that in all conditions of activation, the level of TNF-α were significant over produced in LR-MDS compared to HD **(Table 4-2)**. TNF-α concentration rates were 15-20-fold higher in LR-MDS compared to HD and around 5-10 times higher compared to HR-MDS after anti-CD3+CD28 co-activation. There was no significant response noted for TNF-α secretion after complement treatment in $CD4^+$ T-cells of LR-MDS **(Figure 4-11)**.

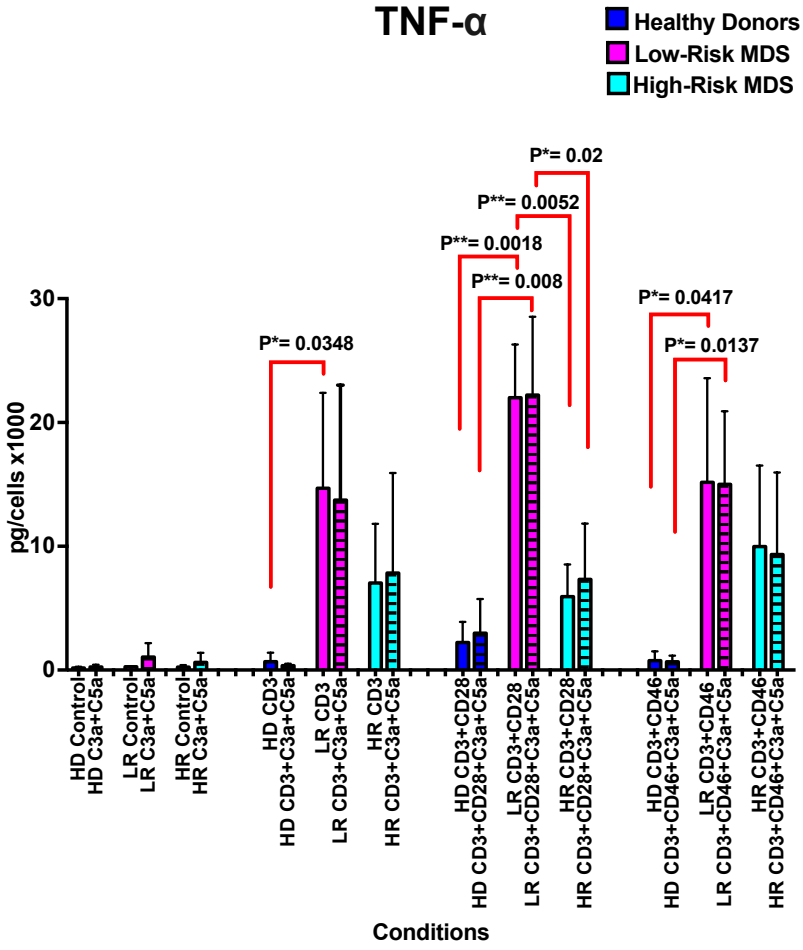


Figure 4-11: $CD4^+$ T-cells pro-inflammatory (TNF-α) secretion patterns is higher in LR-MDS patients.
Isolated naïve $CD4^+$ T-cells from PBMCs of healthy (n=3), low-risk MDS (n=3) and high-risk MDS (n=3) donors were maintained non-activated or activated with CD3 alone, (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) 50nM C3a and 50 nM C5a for 24 h. Then the supernatants were collected, and the concentrations of secreted cytokines were measured. Error bars indicate SD of three experiments. Data are representative of three experiments with similar results.

Finally, IL-10 secretion from activated $CD4^+$ T-cells was significantly over-secreted in LR-MDS patients after anti-CD3, anti-CD3+CD28 and anti-CD3+CD46 activation **(Table 4-2).** $CD4^+$ T-cells from LR-MDS cells were secreted IL-10 (32-35 times) more than of HD. As expected, anti-CD3+CD46 co-activation increased IL-10 secretion in HR-MDS as well as LR-MDS cells, with a slightly response in HR-MDS and no response in LR-MDS following complement treatment **(Figure 4-12).**

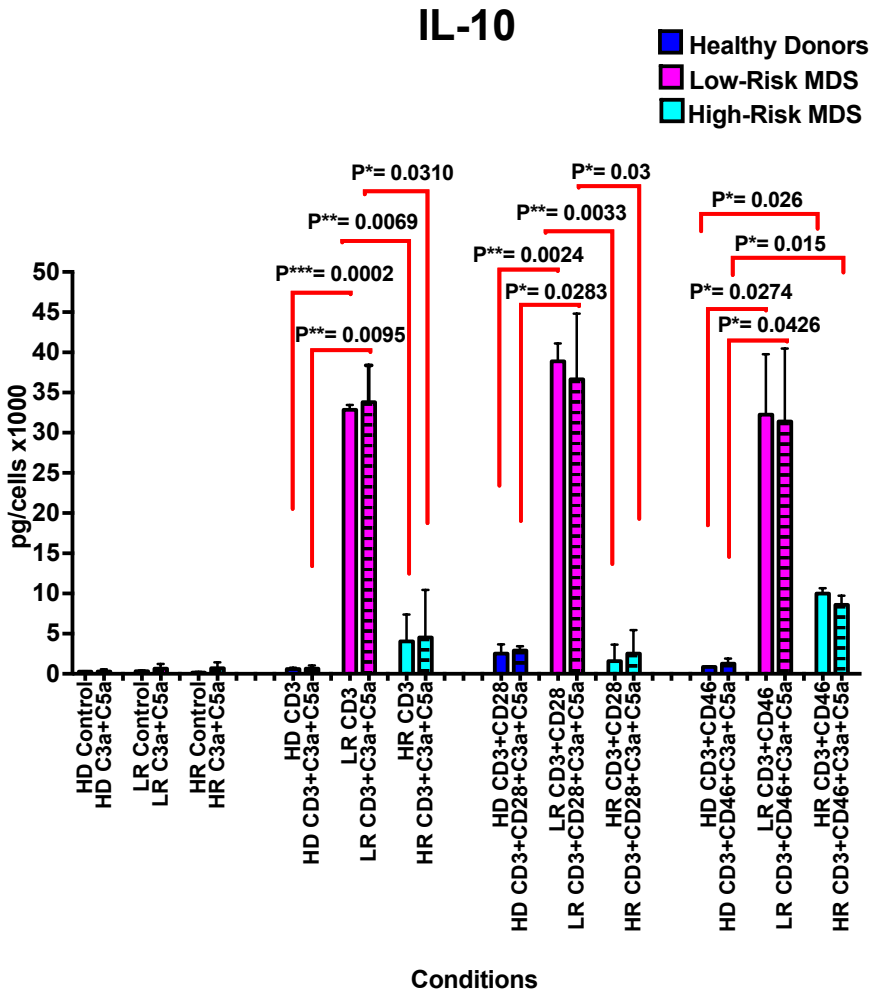


Figure 4-12: Isolated $CD4^+$ T-cells pro-inflammatory (IL-10) secretion patterns is over-secreted in LR-MDS patients.
Isolated naïve $CD4^+$ T-cells from PBMCs of healthy (n=3), LR-MDS (n=3) and HR-MDS donors (n=3) were maintained non-activated or activated with CD3 alone, (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of C3a and C5a 50 nM for each (for 24 h). Then the supernatants were collected, and the concentrations of secreted cytokines were measured. Error bars indicate SD of three experiments. Data are representative of three experiments with similar results.

4.3 Discussion

In this chapter I observed both mTOR and other TGF-β signaling Pathway-Associated Proteins (pSMAD2, pSMAD3, pSMAD4) were significantly reduced in HR-MDS patients relative to HD and LR-MDS patients. This might be associated with a defect in the $CD4^+$ T-cell signalling pathway. TGF-β is an important physiologic regulator of cell growth and differentiation. Previous work has revealed the roles of C3a and C5a, which bind to their receptors on T-cells. Kolev, Dimeloe, Le Friec et al. (2015) have provided evidence for a function of the C3b-binding protein CD46 in the enhancement and sustainment of signalling to mTORC1 as part of the mechanisms by which complement components are able to regulate functional characteristics of T-cells (Boothby et al., 2015). Other studies have demonstrated that conventional and regulatory T-cell subsets are altered by complement components acting directly on T-cells.

With reference to cytokine role, there were significant increases in the levels of IL-10, IL-17, IL-18, IFN-γ and TNF-α, and high levels of IL-2 in LR-MDS patients compared to HD and HR-MDS patients. IL-1RA and IL-6 were the only cytokines significantly elevated in HR-MDS patients relative to HD and LR-MDS patients. CD46 shares a phenotype with Tr1 cells; it can induce low IFN-γ and high IL-10 in naïve CD3-activated $CD4^+$ T-cells (Cardone et al., 2010). Therefore, CD46 plays a significant role in the regulation of adaptive immunity regulation and its deficiency has associations with autoimmune diseases and chronic inflammation (Kemper and Atkinson, 2007). The principal Th1 cytokine, IFN-γ, is produced by $CD4^+$ T-cells (Abramson et al., 2001). However, it was interesting to observe that the levels of IFN-γ secretion in LR-MDS was not change following CD3+CD46 co-activation plus C3a+C5a treatment. These results suggest a negative correlation in LR-MDS patients: IFN-γ secretion is increased by anti-CD3, anti-CD3+CD28 and anti-CD3+CD46 activated $CD4^+$ T-cells and no effect by complement treatment. Several types of immune cells secrete IL-6, including Th2 lymphocytes. Moreover, IL-17A acts on a variety of cells to induce cytokine such as IL-6 and TNF-α (Fossiez et al., 1996). $CD4^+$ T-cells from patients with LR and HR-

MDS have higher IL-6 concentration than HD when non-activated and also after CD3, CD3+CD28 and CD3+CD46 stimulation. High levels of IL-6 in HR-MDS patients could account for the dysregulation of $CD4^+$ T-cell activation and induction. Moreover, complements have an important influence in the promotion of the function, activation and induction of $CD4^+$ T-cells in MDS patients.

IL-2 is produced mainly by activated Th1 cells and is an important immune response and homeostasis regulator. It also plays a pivotal role in the downregulation of immune response, for example its absence often results in severe autoimmunity (Malek, 2008). This could be related to deleterious biomaterial interactions, which promote activation of signaling pathways inducing pro-inflammatory cytokine secretion in $CD4^+$ T-cells.

TNF-α is a pro-inflammatory cytokine produced by Th1 cells that promotes several autoimmune diseases. IL-10 inhibits the production of IFN-γ, TNF-α and IL-2. A study by Mallat et al. has presented findings to verify the anti-inflammatory and immunosuppressive activity of IL-10 (Mallat et al., 1999). Nevertheless, it is still uncertain whether high plasma levels of IL-10 are to be measured as a marker of anti-inflammatory patterns or conversely whether they are counter-regulatory outcomes related to primitive pro-inflammatory patterns (Heeschen et al., 2003). Another major source of IL-10 is $CD4^+$ Treg cells, thus the increased IL-10 secretion in LR-MDS patients' co-activated T-cells could reflect an effort of these cells to shift from IFN-γ to IL-10 secretion; from a Th1 to a Treg phenotype (progressing the disease from LR to HR-MDS). However, the high levels of IFN-γ observed in the same condition imply that this transition, which is essential for the regulation of innate and adaptive immune response, does not occur.

The findings here provide the first evidence that complement regulates $CD4^+$ T-cells by directly affecting the fate and properties of these cells. The results establish that defects in the TGF-β signalling pathway proteins (pSMAD2, pSMAD3 and pSMAD4) as well as mTORc and no or low response to complement treatment contribute to $CD4^+$ T-cell dysfunction and

irregularities in cell fate response in MDS patients. Therefore, complements, TGF-β signalling pathway proteins, and mTOR represent potential therapeutic targets for improving MDS therapy. Finally, no single treatment is effective for all patients with MDS due to its biologic heterogeneity. With more detailed knowledge of cytokine signalling cascades, together with technological developments in genomics and proteomics, the future treatment of MDS may lie in combination therapies tailored for appropriate biological effectors.

4.4 Perspective

While this data suggests a possible connection between complement and T-cells, the cytokine data is not sufficient to make a definitive conclusion. This was the reason for carrying out the phenotyping and WB assays before and after anti-CD46 stimulation (as shown in the previous chapter). Furthermore, the results of the mTOR and TGF-beta proteins levels following complement support the above hypothesis. Jointly, this chapter demonstrate that the mTORc protein level in HR-MDS is reduced and does not change in response to complement components or to receptor stimulation, and neither does the level of Akt. This may prevent $CD4^+$ T-cells polarizing towards pro-inflammatory T-cells (Th1 or Th17) therefore averting an effective immune-surveillance against malignant clones. Lack of response to complement related co-stimulation and increase in C5aR2 expression indicate a potential mechanism for Treg expansion in MDS. Overexpression of pro-inflammatory cytokines is strongly associated with LR-MDS. These findings may lead to novel therapeutic targets being identified in MDS, although further studies are needed on a larger cohort of patients. Moreover, these data show that complement components may modulate cytokine and chemokine-induced differentiation and activation of $CD4^+$ T-cells, possibly through TGF-β regulation.

To confirm this I will estimate the gene expression of T-cells before and after anti-CD46 stimulation following two conditions i.e. in the presence or absence of complement (C3a+C5a).

Chapter 5. Results III

Gene expression profile of T-cells after complement pathway activation

5.1 Introduction

In this chapter I have used an analytical approach to investigate the differential gene expression profiles of T-cells in MDS and HD following stimulation with anti CD46 with or without complement components C3a+C5a. Whiles I focused on the specific complement related pathways in the previous chapters, in this chapter I wanted to determine the involvement of other pathway, if any, following the stimulation of Tconv or Tregs $CD4^+$ T-cells with anti-CD46 using gene expression profiling.

5.2 Expression microarray data analysis

The HT 2.0 arrays were first scanned using a GeneChipR 3000 Scanner (7G upgrade) to capture images (DAT files). The raw data from the DAT file is converted to CEL files (based on intensities). ITERPLIER normalization was used to generate the CHP file. The outcomes from microarray analysis were first uploaded into Qiagen's Ingenuity pathway analysis (IPA) system for core analysis. IPA is a bioinformatic tool, which can analyse the gene expression patterns using a build-in scientific literature based database. IPA was performed to provide some new knowledge and identify signalling pathways, diseases and functions, and gene networks that are most significant to microarray outcomes and to categorise differentially expressed genes in MDS. In case of mutations, the gene expression in MDS patients with the mutant was compared with that of healthy donors. Heatmap analysis was used to demonstrate the expression patterns of these differentially expressed genes. The data was filtered using IPA p value ≤ 0.05 to create a list of significantly up or down regulated pathways.

5.3 Results

5.3.1 Gene Expression profile of the healthy donors

Ingenuity Pathway Analysis (IPA) highlighted several gene sets that are significantly down regulated or up-regulated in T-conv cells in HD **(Table 5-1).**

Table 5-1: Genes that are downregulated and upregulated in the T-conventional health donors

Condition (Healthy Donors)	FDR q-Value	Significant Up-regulated Genes	Significant Down-regulated Genes
CD46 Stimulated vs Unstimulated T-conventional cells	< 0.05	DARS TCP1 RUNX3	MIR3675 MIR4446

The most significant gene sets that are down- and upregulated were compared between CD46 stimulated vs. unstimulated in the Tconventional health donors.

The heatmap in **Figure 5-1** shows a comparison of the significantly overexpressed genes between CD46 stimulated vs. unstimulated cells ($p < 0.05$).

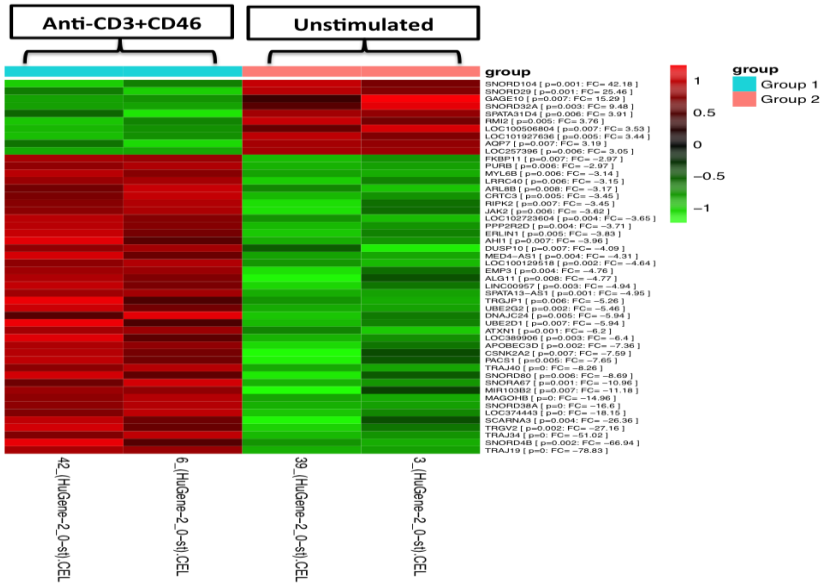


Figure 5-1: Heatmap depicting up-regulated and down-regulated genes in healthy donors
Heatmap of the topmost up-regulated and the topmost down-regulated genes in Tconv cells from anti-CD3+CD46 stimulated (n=2) vs. unstimulated (n=2) healthy controls, as determined by micro-array analysis. Expression levels were listed in order of significance (P<0.05). Higher levels of expression are displayed in **red** and the lower levels in **green**.

The network analysis for CD46 stimulated versus unstimulated Tconv cells in HD was created, the data were normalised and the genes that were upregulated identified. IPA software was used to analyse the data (QIAGEN Bioinformatics). There were several activation signalling pathways (highlighted in red) that were significantly upregulated after stimulation with CD46 (**Figure 5-2**) and these included the genes below in the **Table 5-2** with their corresponding p-values (all p-values are adjusted p-value to account for false discovery rate).

Table 5-2: Upregulated pathways after anti-CD46 stimulation in healthy donors

Gene name	FDR q-value
Complement system	5.26×10^{-2}
PI3K/AKT signalling	5.28×10^{-2}
PAK signalling	2.18×10^{-2}
Tec Kinase signalling	4.08×10^{-2}
TNFR2 signalling	4.95×10^{-2}
HMGB1	6.61×10^{-2}
Role of pattern recognition receptors in recognition of bacteria and viruses	6.45×10^{-2}
Notch signalling	6.92×10^{-2}
Molecular mechanisms of cancer	6.04×10^{-2}
Mitochondrial dysfunction	3.23×10^{-2}
Retinoic acid mediated apoptosis signalling	2.38×10^{-2}
PTEN signalling	4.87×10^{-2}
Granulocyte adhesion and diapedesis	3.40×10^{-2}
Agranulocyte adhesion and diapedesis	3.06×10^{-2}
Agrin interactions at neuromuscular junction	3.80×10^{-2}
Regulation of actin-base of motility by Rho	5.75×10^{-2}
Actin nucleation by ARP-WASP complex, purine ribonucleosides degradation to ribose-1-phosphate	2.76×10^{-2}
GDP-glucose biosynthesis	4.04×10^{-3}
Glucose and glucose-1-phosphate degradation	4.04×10^{-3}

Of the above activation signalling pathways the ones with the highest adjusted p-values were have been shown in bold. The complement system and the PI3K/AKT signalling pathway which were also upregulated have been discussed in the previous chapters, which are also of importance to this thesis.

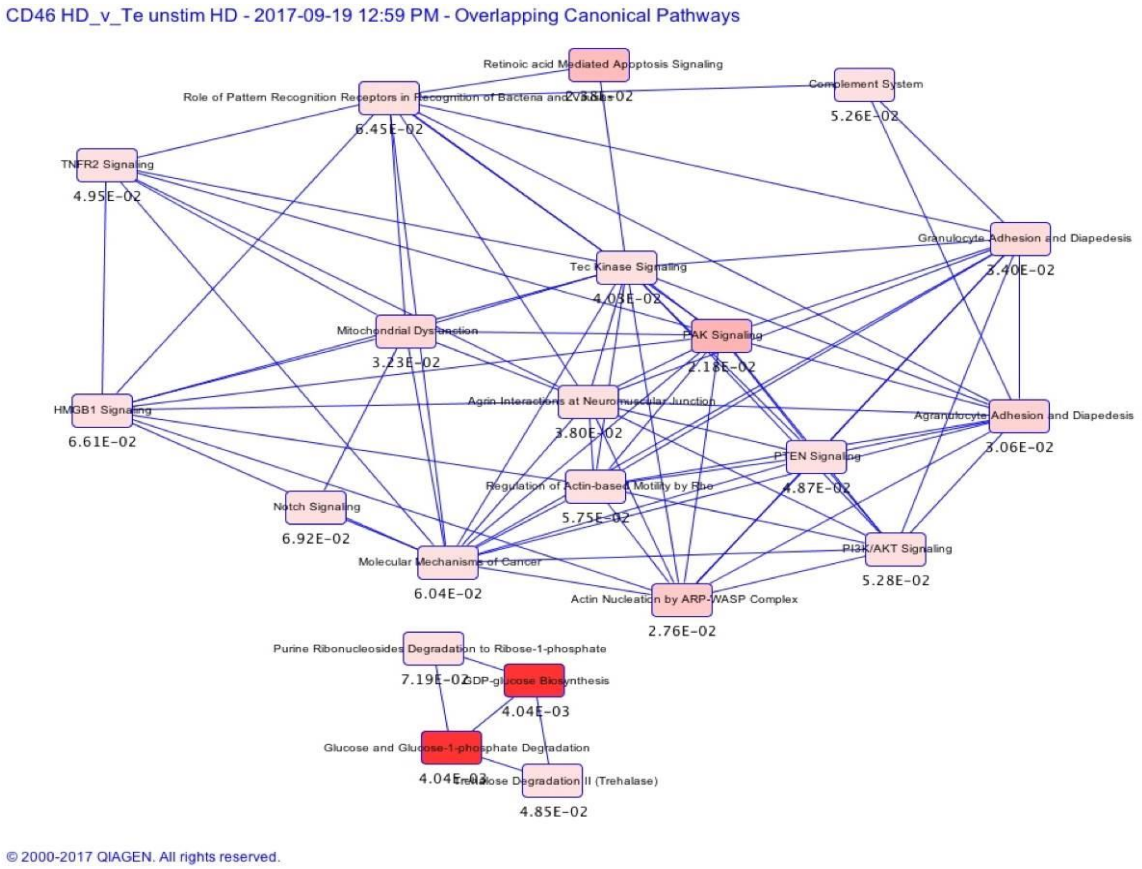


Figure 5-2: The activation signaling pathways in the Tconventional health donors.

The most significant activation signaling pathways (**highlighted in red**) were increased by anti-CD46 stimulation in Tconventional healthy donors.

IPA of the functions of these genes **(Figure 5-3)** showed that they are possibly involved in increasing the activity of T-cells movement, proliferation, infiltration, development and migration. The stimulation with anti-CD3+CD46 increased the expression of the following genes: *BCL2L11, CFH, PM2, PLA2G10, ITGA3, TNSFSF10* and *MAP2K4* (highlighted in red), but decreased the expression in *CD6* and *C5aR1* (highlighted in green). The reduction of *C5aR1* after anti-CD3+CD46 stimulation inhibited T-cells development, cellular infiltration and the cell proliferation as shown by the dotted blue lines. Of the genes that were increased after anti-CD3+CD46 stimulation, *CFH* inhibited cellular infiltration, while *TNFSF10* inhibited both the cellular infiltration and proliferation of T-cells. On the other hand, *PM2* enhanced the T-cells proliferation (shown by the doted orange lines); *MAP2K4* enhanced T-cells development and proliferation; *ITGA3* enhanced both T-cells movement and migration while *PLA2G10* led to an enhanced T-cells movement, cellular infiltration and migration. Both *BCL2L11* and *CD6* which were increased and decreased respectively had no functional effect on T-cells.

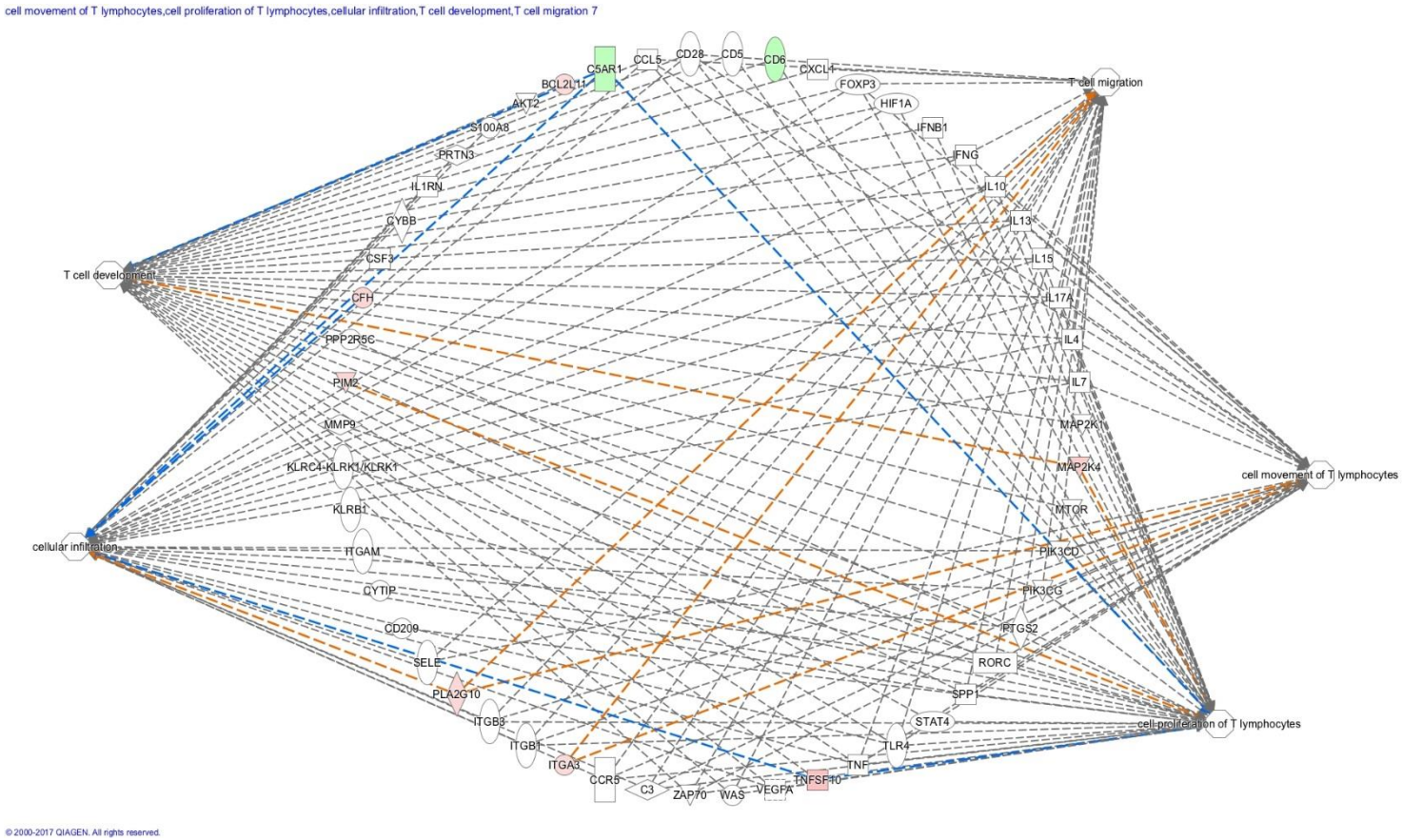


Figure 5-3: Significant genes functions of Tconv cells in HD.
The stimulation of Tconv cells with anti-CD3+CD46 vs unstimulated cells resulted in increased ***BCL2L1, CFH, PM2, PLA2G10, ITGA3, TNSFSF10*** and ***MAP2K4*** (**highlighted in red**) and decreased CD6 and C5aR1 (**highlighted in green**). Stimulated functions marked by orange and the inhibiting actions in blue.

5.3.2 Differences between MDS and HD donors after anti-CD46 stimulation in T conventional cells

In MDS vs HD after anti-CD46 stimulation of T conventional cells, IPA showed significant up-regulation in T-conventional cells for 10 genes whiles 4 genes were downregulated as listed in **Table 5-3.**

Table 5-3: Genes that are downregulated and upregulated in the T-conventional in MDS vs HD after anti-CD46 stimulation.

Condition	FDR q-value	Significantly upregulated genes	Significantly downregulated genes
Anti-CD46 stimulated MDS vs Anti- CD46 stimulated HD Tconventional	<0.05	*TES* *CLPX* *C10orf35* *SLAMF6* *BNIP2* *ARFGEF2* *DNAJC16* *LUC7L3* *MMADHC* *TMEM167A*	*GPR65* *MIR4290* *XXYLT1-AS2* *LOC101929626*

The heatmap in **Figure 5-4** shows a comparison of the significantly overexpressed genes between CD46 stimulated in HD (n=2) vs MDS patients (n=3). It can be seen that the genes that were upregulated in HD were downregulated in MDS patients and vice versa.

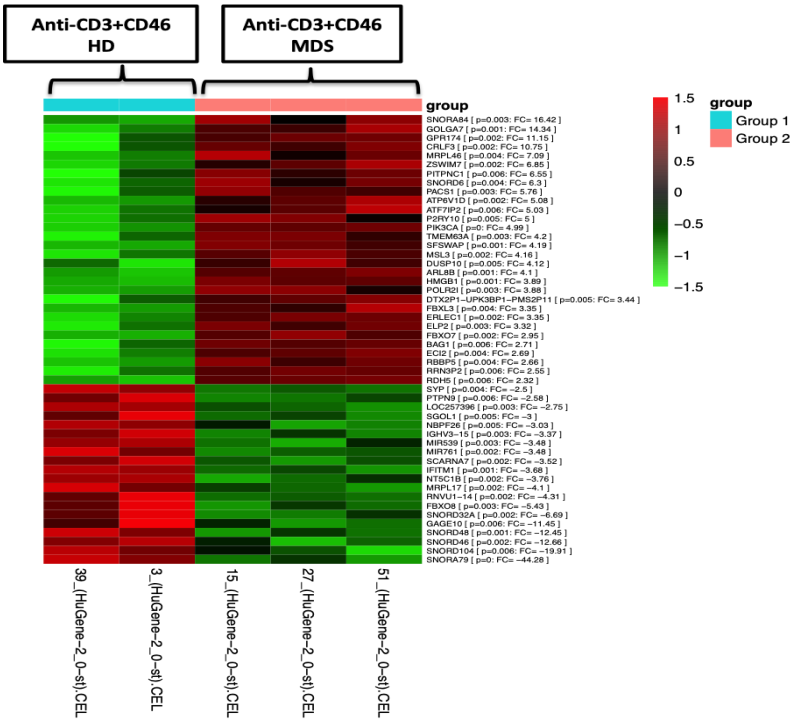


Figure 5-4: Heatmap depicting up-regulated and down-regulated genes in anti-CD3+CD46 stimulation in MDS vs HD. Heatmap of the topmost up-regulated and the topmost down-regulated genes in Tconventional cells from anti-CD3+CD46 MDS (n=3) vs anti-CD3+CD46 HD (n=2), as determined by micro-array analysis. Expression levels were listed in order of significance (P<0.05). Higher levels of expression are displayed in **red** and the lower levels in **green**.

In the top signalling pathways, rheumatoid arthritis pathway and Triggering receptor expressed on myeloid cells 1 (TREM-1) pathway came out to be the top two most significant pathways in MDS patient after anti-CD3+CD46 stimulation **(Figure 5-5 and 5-6)**.

In the rheumatoid arthritis pathway of MDS patients there was reduction in four downstream molecules (*NF-kB, IL-23* which polarized towards Th17, antigen presentation (*TLR* and *MHC II*) and *Fas* receptor (also known as an apoptotic antigen) compared to HD. The reduced *IL-23* pathway could potentially prevent polarisation toward Th17 phenotype while reduced Fas receptors expression also suggests that MDS cells do not undergo apoptosis as their respective ligand would be unable to bind to any receptor. This may suggest that MDS patients' T-cells after anti-CD3+CD46 stimulation although may not go through FAS

mediated apoptosis, however, cannot proliferate and may not polarised toward Th17 cells as is the case in HD **(Figure 5-5)**.

In the TREM-1 signalling pathway there was a reduced expression of *NF-kB,* TLR and an increased expression of *NLR, Caspase 1 and 5. NF-kB* is important in secretion of proinflammatory cytokines, hence a reduction of these genes will cause a reduction in the associated downstream cytokines. TLR reduction will also potentially affect the secretion of the following cytokines TNF-α, IL-β, IL-6, IL-10 and GM-CSF in MDS patients compared to HD. Increased expression of NLR and Caspase (inflammasome pathway) will possibly lead to increased inflammasome activity which may contribute to the proliferation pressure and subsequent disease progression as has been previously suggested (Sallman et al., 2016**) (Figure 5-6)**.

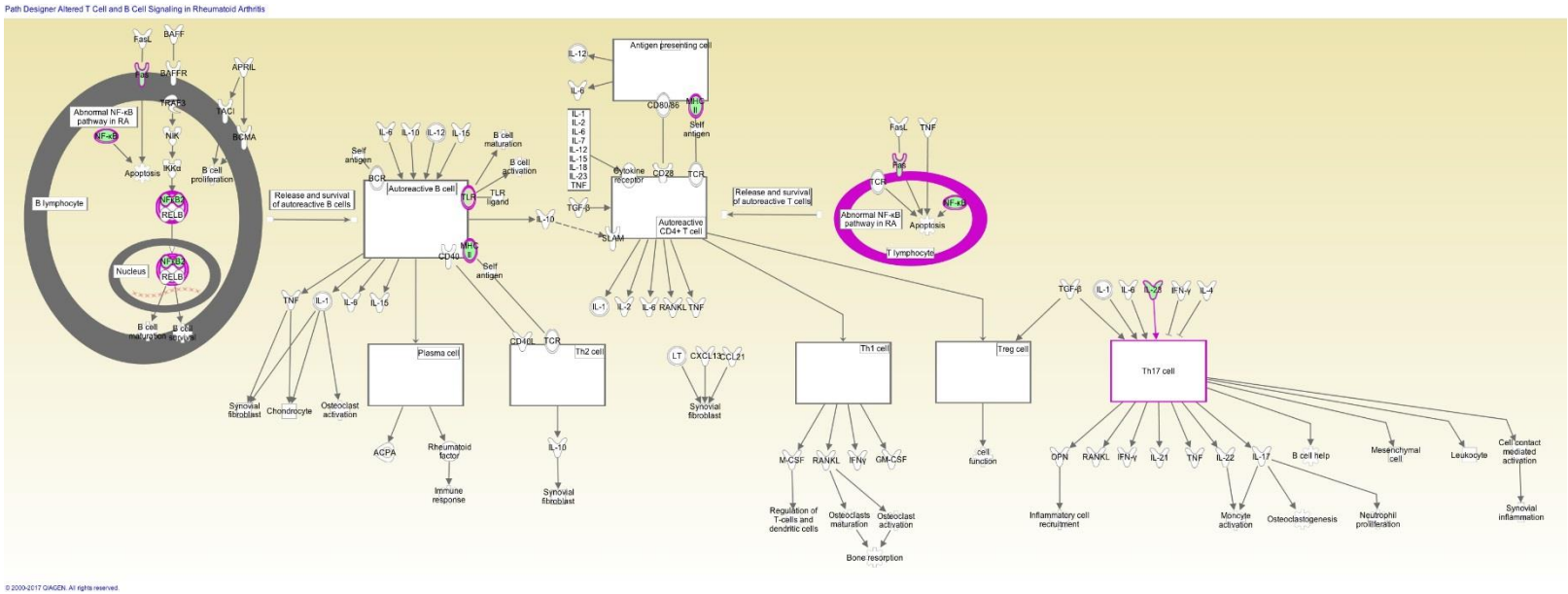


Figure 5-5: Rheumatoid arthritis pathway in MDS patients

The molecules in **green** are the significantly reduced ones in MDS patients compared to HD.

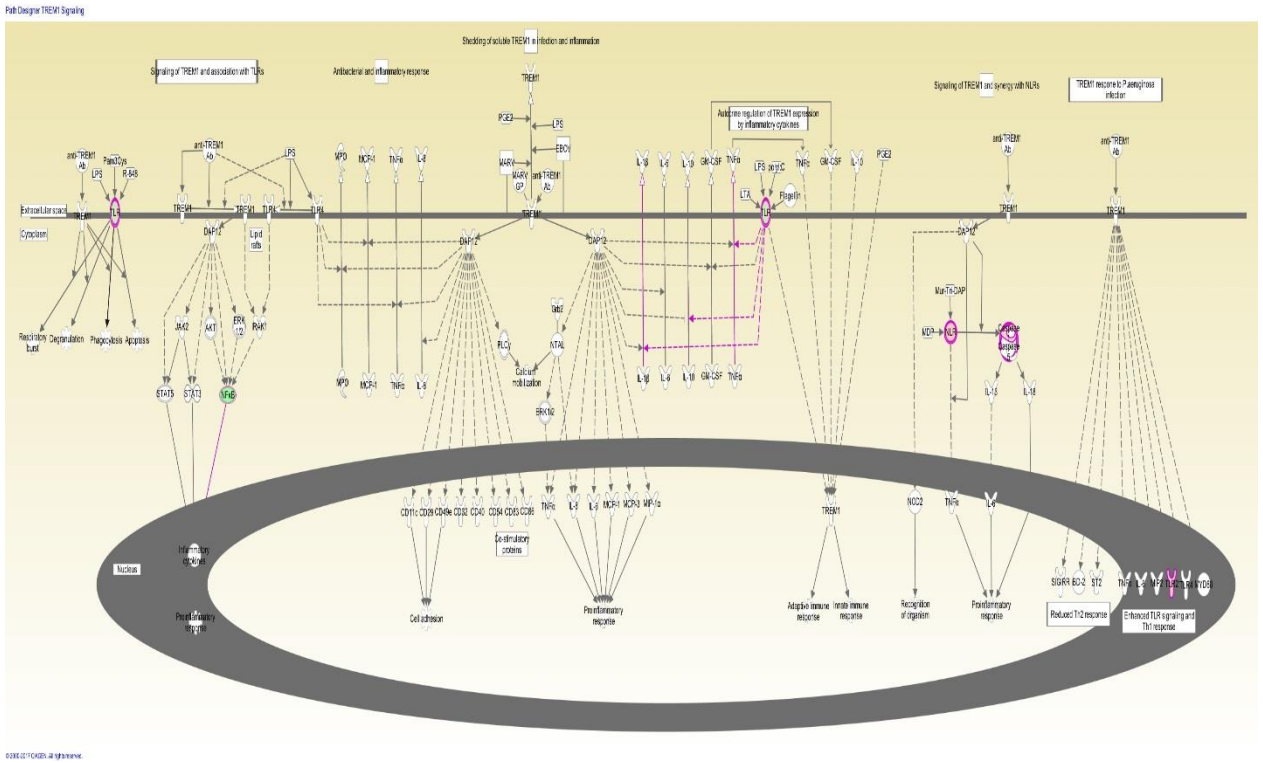


Figure 5-6: TREM1 pathway in MDS patients' vs healthy donors after anti-CD3+CD46 stimulation.
The **green** is inhibition and the **red** is increased expression.

A method to predict the upstream regulators and their target molecules revealed that several of them were significantly reduced in MDS patients compared to HD after anti-CD46 stimulation **(Figure 5-7) (Table 5-4)**, of which the three main regulators that are involved in MDS include *FOXO1, POU5F1, STAT5a /b3*. In a normal expression *POU5F1* inhibits *CASP1* and *CASP6* (in red) but in its reduced expression it appears to have instead inhibited and reduced *FAS*, *PYCARD* and *PAMAIP1* expression; this however, resulted in increased *CASP1* and *CASP6. STAT5a/b* is another possible regulator that did not change significantly but at the same time it had an increase effect on *WDR3, PDE4B* and *MAF*. Also, a marginal change in *FOXO1* may possibly lead to an increase and decrease expression of *CCL20* and *PAMAIP1* respectively.

Table 5-4: Significant upstream regulators with their corresponding target molecules

Upstream regulator	**Target molecules**	**p-value overlap**
RB1	CASP6, FAS, MAF, RRM2	0.000717
CD3	BCL6, CCL20, CCR4, CD74, FAS, MAF, PDE4B, TLR2	0,000724
RELB	CIITA, NFKB2	0,00233
REL	CIITA, NFKB2, PMAIP1	0.00244
IRF8	BCL6, CIITA	0.00566
miR-155-5p (miRNAs w/seed UAAUGCU)	CASP1, CCL20, IL23A	0.00919
POU5F1	BNIP2, CASP1, CASP6, FAS, PMAIP1, PYCARD	0.0152
STAT5a/B	MAF.PDEA4B, WDR3	0.0179
FOXO1	CCL20, PMAIPI.SGK1	0.0179
RELA	CCL20, CIITA, NFKB2P, PDE4B, TLR2	0.018
PRKACB	PDE4B	0.02
SMC3	HLA-DRB1	0.02

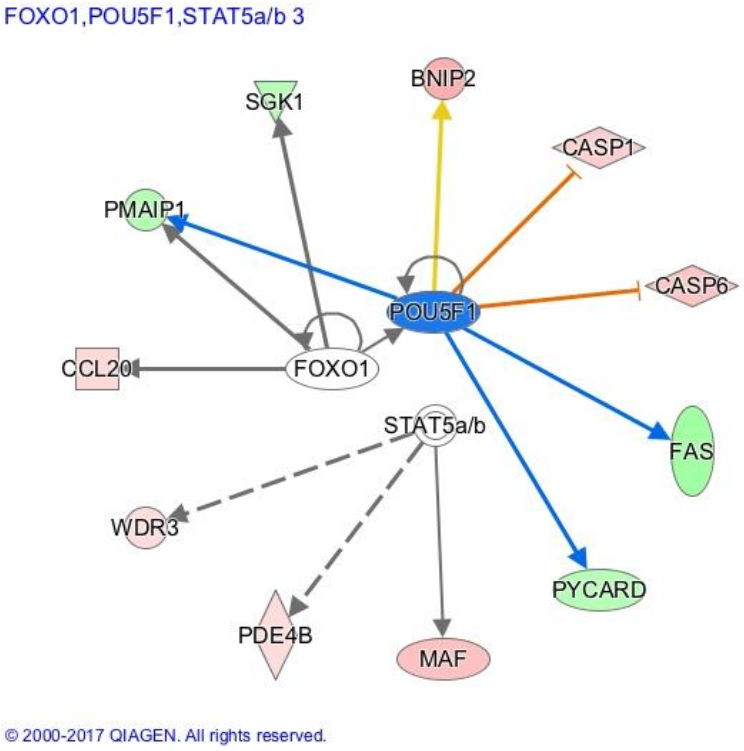


Figure 5-7: Effect of FOXO1, POU5F1, STAT5a /b3 on their target molecules.
These three major upstream regulators were reduced in MDS patients compared to HD when stimulated with ant-CD46.

5.3.3 Differences between MDS and HD donors after anti-CD46 stimulation in the presence of complement

As shown above, anti-CD46 stimulation resulted in reduced NF-kB expression in MDS Tconv. I then wanted to assess the effect of adding complement components in addition to anti-CD46 to deduce whether it will improve *NF-kB* expression. Following this stimulation, I identified a few genes which were up or down regulated in MDS Tconv as shown in **Figure 5-8 and table 5-5.** Nevertheless, these genes do not appear to have important function in T-cells.

However, by adding complement, *NF-kB* expression was rescued in MDS patients to levels similar to that observed in HD but the downstream effect on IL-17A pathway which is c-JUN and IkBzeta was reduced making this IL-17A pathway lower in MDS patient compared to HD (**Figure 5-9)**.

Table 5-5: Genes that are downregulated and upregulated in the T-conventional in MDS vs HD after anti-CD46 plus complement stimulation.

Condition	FDR q-value	Significant upregulated genes	Significant downregulated genes
Anti-CD46+complement MDS vs Anti-CD46+complement HD Tconventional	<0.05	*MT1F* *PPDPF* *NDUFAF2* *NUP85* *TAP9B*	*SLC25A46* *LRCH1* *RHOC* *FLNA* *LOC105369140* *PSMB7* *PSMB3* *RRP7A*

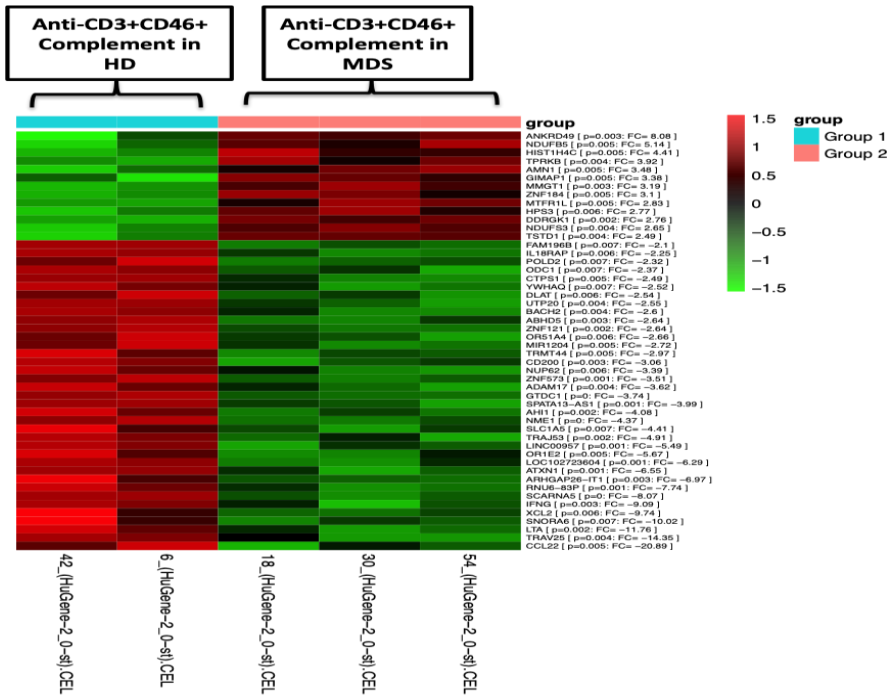
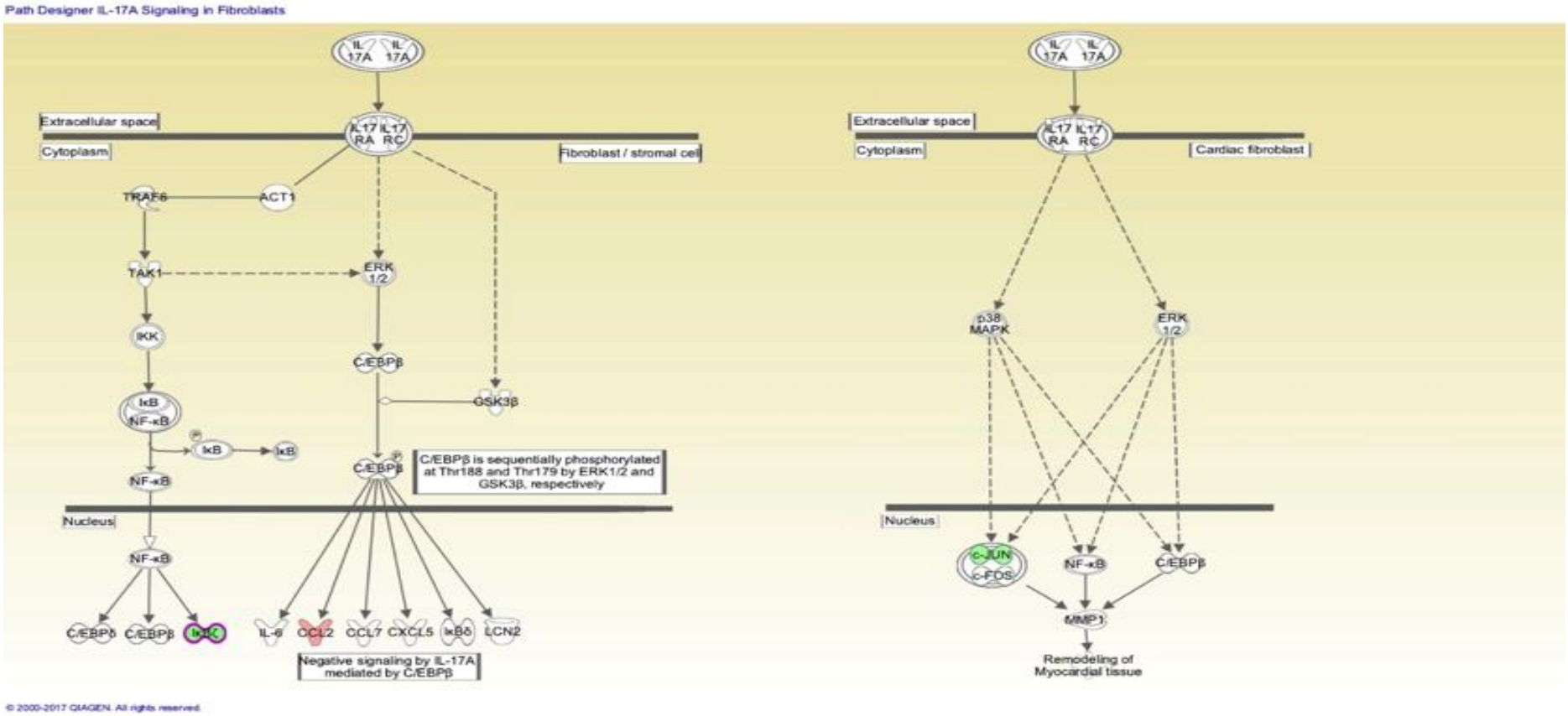


Figure 5-8: Heatmap depicting up-regulated and down-regulated genes in anti-CD3+CD46 stimulation with complement in MDS vs HD.
Heatmap of the topmost up-regulated and the topmost down-regulated genes in Tcon cell from MDS (n=3) vs HD (n=2), as determined by micro-array analysis. Expression levels were listed in order of significance (P<0.05). Higher levels of expression are displayed in **red** and the lower levels in **green**.

Figure 5-9: Signaling pathway in MDS patients' vs healthy donors after anti-CD3+CD46 stimulation with complement.
The **green** is inhibition and the red is increased expression.



5.4 Discussion

In this chapter, microarray has been applied to identify differentially expressed genes in the MDS vs HD after stimulation with anti-CD46 in the presence or absence of C3a+C5a treatment. I have used IPA analytical approach to investigate the differential gene expression profiles of T-cells in MDS and HD. The inflammation and immune response, cytokine signaling, cellular growth and movement and function, were significantly activated suggesting their critical roles in MDS.

I compared the gene expression of anti-CD46 stimulation vs unstimulated Tconv cells in HD. I found that *DARS, TCP1* and *RUNX3* were upregulated when cells were stimulated with anti-CD46 while genes such as *MIR3675* and *MIR4446* were downregulated. In relation with the findings and discussions in the previous chapter I found that the complement system and the PI3K/AKT signalling pathway may have a role in upregulating Tconv cells after stimulation with anti-CD46.

CD46 has a role in T-cell function by upregulating and downregulating some genes that have downstream effect on T-cell proliferation, migration and development and infiltration and movement. For example, decreased *C5aR1* has been shown to affect T-cell development, infiltration and proliferation.

The possible differences in gene expression between HD and MDS patients were investigated in this chapter. After anti-CD3+CD46 stimulation, two major pathways, the rheumatoid arthritis and Triggering receptor expressed on myeloid cells-1 (TREM-1) pathways were detected and significantly activated. In the rheumatoid arthritis pathway of MDS patients there was reduction in four downstream factors/molecules: *NF-kB, IL-23,* antigen presentation *(TLR* and *MHC II*) and *Fas* receptor (also known as an apoptotic antigen). *NF-kB* has a major role in many downstream effects such as the induction or proinflammatory cytokines and enhancement of apoptosis. Increased activity of NF-kB has been shown in MDS (Okubo et al., 2017, Kerbauy et al., 2005). A decrease in *NF-kB* following anti-CD46 stimulation suggests that all the downstream effects such as cytokine induction and apoptosis may be impaired. Several reports show that NF-κB inhibition may be a useful therapeutic target in tumour cell death or growth inhibition (Braun et al., 2006, Breccia and Alimena, 2010). Here, I have shown that despite stimulation with anti-CD46, MDS T-cells do not upregulate their *NF-κB* expression which prevent proliferation and impaired their function.

The reduced *IL-23* pathway could potentially prevent polarisation toward Th17 phenotype while reduced *Fas* receptors expression also suggests that MDS cells do not undergo

apoptosis as their respective ligand would be unable to bind to any receptor. This may suggest that MDS patients' T-cells after anti-CD3+CD46 stimulation although may not go through Fas mediated apoptosis, however, cannot proliferate and may not polarised toward Th17 cells as is the case in HD. A reduction in *Fas* receptor expression by anti-CD46 stimulation may suggest that in MDS patients there is the potential for reduced apoptosis as receptor-ligand interactions may be impaired.

Similar to what was observed in the rheumatoid arthritis pathway, there was a reduction in *NF-kB* expression in the TREM-1 signalling pathway. Engagement of TREM-1 binding to its receptor (although unknown) amplifies the Toll-like receptor (*TLR*)-initiated responses to microbes and triggers the downstream synthesis of pro-inflammatory cytokines. It is therefore possible that the reduced *TLR* expression will impair the TREM-1 pathway and affect its downstream induction products. Engagement of TLRs by their specific ligands leads to the activation of transcription factors that cooperatively regulate the expression of IFNs and other pro-inflammatory cytokines and chemokines (Ganan-Gomez et al., 2015). Between 40–80% of MDS patients have been shown to have overexpressed levels of certain TLR and their signal transducers (Maratheftis et al., 2007, Kuninaka et al., 2010) and hence the inhibitory action of anti-CD46 on TLR is a very significant finding as this may lead to under expression of multiple TLR downstream signalling mediators such as the secretion of the following cytokines TNF-α, IL-β, IL-6, IL-10 and GM-CSF.

An increased expression of *NLR, Caspase 1 and Caspase 5* following anti-CD46 stimulation may lead to increased inflammasome activity and an increased *IL-1β* secretion. Finally, only Interleukin-17A (IL17A) signaling pathway in MDS patients' vs healthy donors after anti-CD3+CD46 stimulation with complement (C3a+C5a) treatment is significantly activated. It showed the importance of complement in rescuing *NF-kB* expression in MDS patients following anti-CD46 stimulation which may improve T-cell function in these patients and restate an effective immune-surveillance.

5.5 Perspective

For future work it is important to employ additional techniques such as DAVID, Gene-mania, KEGG and other gene ontologies analytic tools to look for connections/pathways as these have been shown to give a better understanding of the statistical significance of the pathways. Gaining a better understanding of the basic nature of the MDS by determining critical molecular differences between MDS and HD samples helps accelerate the identification of

drug targets and diagnostic or prognostic markers. Our results may help to illuminate the molecular aspects of MDS and to develop potential therapeutic targets of the disease. Thus, creating new treatment paradigms targeting these networks in their entirety, rather than single proteins, could be necessary for controlling and treating MDS.

5.6 Limitations

The main limitation of this study is the limited number of participants in each subgroup. Although as expected, the statistical power was reduced due to the small number of samples, these findings could be used in future research on higher number of patients.

Replicating these findings in a larger study is an important future step. Furthermore, the limited sample size probably leads to inflated p-value; therefore it is hard to draw a definitive conclusion on the implicated pathways and genes in our study. A replication using a bigger study is essential using qPCR, targeted sequencing techniques such as MySEQ or PcBio and in vitro experiments for confirmation of up and down regulated genes is essential in future analysis.

Chapter 6. The General Discussion, Future Studies and Limitations

6.1 General Discussion

The complement system is one of the oldest known systems involved in defence against pathogens. It can be activated by pathogen-associated or danger-associated molecular patterns leading to the cleavage of the complement components C3 and C5 into the respectively anaphylatoxins C3a and C5a, as well as the generation C3b and C5b, both opsonins, all of which play various roles in innate immune response and activation of the adaptive arm of the immune system.

Intracellular complements such as C5a through C5aR2 mediated TGF-β pathway, and C5a through C5aR mediated mTOR pathway play a role in driving $CD4^+$ T-cells toward either a regulatory or effector phenotypes. Although these complement components and their receptors (C3aR, C5aR1 and C5aR2 (C5L2)) play essential roles in innate and adaptive immune responses, their contribution in priming $CD4^+$ T-cells in patients with MDS is not clear.

Considering the importance of T-cells and the role of complement in the immune response to pathogens and other haematological malignancies, this work was carried out to investigate the potential role of intracellular complement components C3a and C5a, regulator CD46 and their receptors C3aR, C5aR1 and C5aR2 in inducing $CD4^+$ T-cell polarisation and function in MDS patients. To do this I investigated whether there are differences between MDS and HD in terms of intracellular and surface complement receptors (C3aR, C5aR1 and C5aR2) and complement component (C3a and C5a) and if so, any differential expression between LR and HR-MDS disease.

Furthermore, any potential differences between HDs, LR and HR-MDS in response to complement pathway activator (mainly CD46), a well as the main signalling pathways for polarising $CD4^+$ T-cells including mTOR, and TGF-β signalling and associated proteins were investigated following stimulation with anti-CD46 in both HDs and MDS patients. Finally, I identified additional three pathways which may be differentially expressed in T-cells from MDS patients compared to HDs following Tconv and Tregs stimulation with anti-CD46 (with or without complement components).

In order to achieve this I optimised a protocol in which the PBMCs from the HD were cultures at four different time points (1min, 1hr, 24hr and 48hr) in the presence (test) or absence (control) of anti-CD3±CD46, and the expression of surface and intracellular complement receptors (CD46, C3aR, C5aR1 and C5aR2) assessed using flow cytometry. The 24-hr time point was shown to be the optimum for this study.

The data suggest that intracellular expression of CD46 in both LR and HR-MDS patients were lower than in HD. Lack of available intracellular complement components in MDS T-cells, subsequent lack of signalling through C3aR and C5aR along with upregulation of C5aR2 (C5L2), an alternative receptor for C5a, in MDS patients may contribute in the T-cell dysfunction and lack of polarisation toward Th1 or Th17. Moreover, the contribution of C5L2 in regulating pro-inflammatory and anti-inflammatory responses and, in particular, certain features of the complement signalling pathway, makes it a possible agent for remedying diseases such as MDS.

I have also investigated the potential changes in TGF-β signalling pathway proteins as well as mTORc following stimulation with anti-CD46 antibody. The data demonstrated that the mTORc protein level in HR-MDS is reduced and does not change in response to complement components or to receptor stimulation, and neither do the level of Akt. This may prevent $CD4^+$ T-cells polarizing towards pro-inflammatory T-cells (Th1 or Th17) therefore averting an effective immune-surveillance against malignant clones.

Lack of response to complement related co-stimulation and increase in C5aR2 (C5L2) expression indicate a potential mechanism for Treg expansion in MDS. Overexpression of pro-inflammatory cytokines is strongly associated with LR-MDS. Moreover, these data show that complement components may modulate cytokine and chemokine-induced differentiation and activation of $CD4^+$ T-cells, possibly through TGF-β regulation. These findings may lead to identification of new therapeutic targets in MDS, although need further studies on larger cohort of patients.

The gene expression profile of Tconv following stimulation with anti-CD46+/- complement components has been studied next. Two major pathways, the rheumatoid arthritis and TREM-1 pathways were found significantly activated and different between MDS and HDs. In the rheumatoid arthritis pathway of MDS patients there was reduction in four downstream factors/molecules (*NF-kB, IL-23*, antigen presentation (*TLR and MHC II*) and *Fas* receptor. Anti-CD46 stimulation also fails to induce *IL-23* pathway in MDS T-cells, a pro-inflammatory cytokine that is essential for the survival of IL-17 producing effector cells

I observed a reduced *Fas* receptors expression upon anti-CD46 stimulation. Similarly to what was observed in the rheumatoid arthritis pathway, there was a reduction in *NF-kB* expression in the TREM-1 signaling pathway. An increased expression of *NLR, Caspase1* and *Caspas5* following anti-CD46 stimulation will lead to increased inflammasome activity and an increased IL-1β secretion.

Finally, this works shows the potential importance of complement in $CD4^+$ T-cells polarisation in MDS and this is the first time that this has been investigated and shows encouraging data which would need to be continued in more detail to elucidate the pathway(s) that leads to the lack of complement in T-cells of MDS patients and also identify potential molecules involved which can serve as potential therapeutic targets in MDS in the future.

6.2 Future studies

The role of complements receptor especially C5L2 in the different subclasses of MDS patients and other results obtained in this thesis raised some prospects which can be addressed in future studies. A consistent theme throughout the thesis work was the emphasis on studying the dynamics of biological processes on complement treated T-cells from MDS patients compared to HDs. As suggested by the results, a further direction would be to expand the study and perform deep phenotyping of $CD4^+$ T-cells following the proposed conditions to identify specific subsets of Tregs and T conv which may be affected/polarised in HDs and or MDS patients. This PhD thesis provides a foundation upon which these other works can be built.

Some of the experiments that I will undertake to support this initial evidence include:

1- Blocking C5L2 with anti-C5L2 blocking mAB to determine whether this receptor is directly involved or not. This will also help to elucidate whether the unusual effects of C5L2 in MDS are reversed or not.

2- Using different concentrations of complement components (C3a+C5a) to understand how that changes the fate of T-cells.

3- Blocking upregulated pathways using particular molecules and confirming the up- and down regulating pathway at protein levels by employing Western blot or Flow cytometry.

4- The gene expression pathways need for biological testing to test the predictions (test the findings biologically (e.g, qPCR, sequencing and *in vitro* experiments for confirmation of up/down regulated genes).

5- To employ additional techniques such as DAVID, Gene-mania, KEGG and other gene ontologies analytic tools to look for connections/pathways as these have been shown to give a better understanding of the statistical significance of the pathways.

6- A fundamental issue to address is whether activated T-cells following complement pathway stimulation could reject malignant clones both *in-vitro* and *in-vivo*. Using the established in-house NSG and NSG-SGM3 mouse models will help to address this question.

7- The GEP data suggests another interesting aspect to be investigated, which is to identify genes which could potentially be targeted for therapeutic purpose and restore an effective immune response in MDS patients.

6.3 Limitations

1- In experiments where purified cell fractions were not used there could be the possibility of heterogeneous within the different cell populations from the different patient types (LR-, HR-MDS and healthy donors) thereby making comparability between the different subsets difficult.

2- It was evident that some of the results of T-cell stimulation experiments showed that the response of HR-MDS samples were more closely to that of normal donors than that of LR-MDS samples. It is therefore possible that these may reflect more as features of LR-MDS (e.g. cytopenias) rather than potential mechanisms of progression.

3- I concede that the sample sizes for the different patient types used in this thesis were very small. Any future work will take this into account and increase the numbers to enable greater statistical power. Any future experimental approaches will be largely based on gene expression approach.

4- Most of the genes have unknown functional background information thereby making more difficult to provide a more in-depth explanation of the findings of the data. One solution will be to perform gene sequencing and to increase the sample size to increase the statistical power.

5- Obviously, funding was also a major challenge. However, this preliminary data can be used as a basis for additional grant to expand on this work.

Appendix 1

Intracellular CD46, C3aR and C5aR1 expression

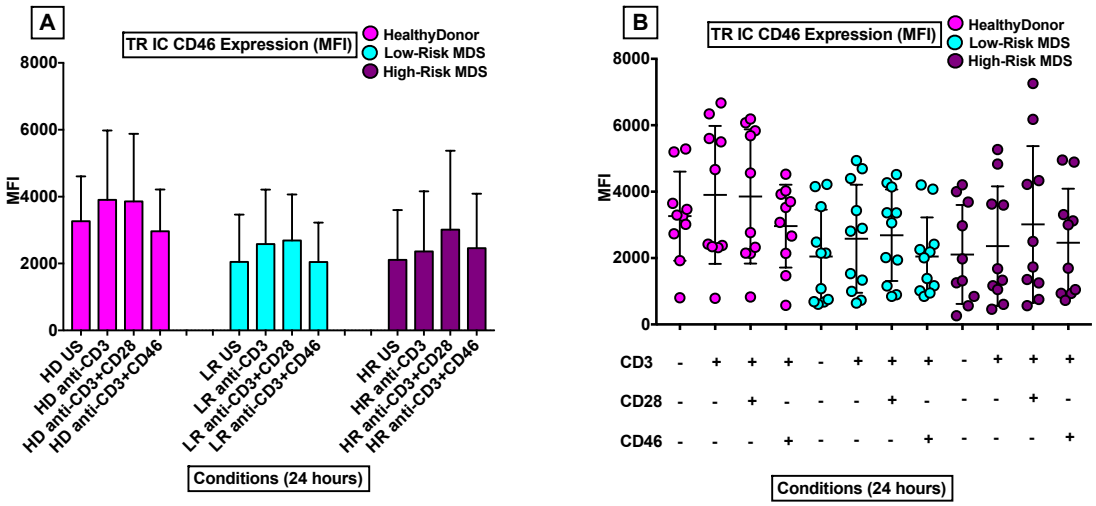


Figure 3-S1. Intracellular MCP (CD46) expression on human Tregs cells.
Cells were activated for 24 h with an anti-CD3, anti-CD3±CD28 and anti-CD3±CD46 antibody. Intracellular CD46 expression in Tregs was measured at the indicated time point. The results are representative of thirty (n=30) experiments and the data show mean ± SD derived from healthy donor (n=10), low-risk MDS (n=10) and high-risk MDS (n=10) experiments.

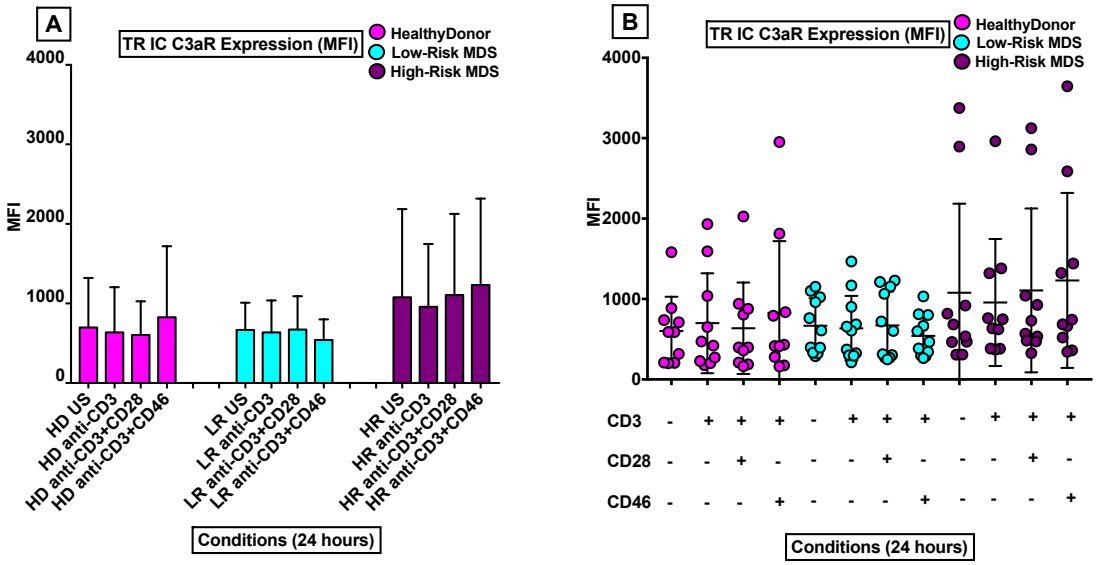


Figure 3-S2. Expression of intracellular C3aR in human Treg cells by flow cytometry analysis (FACS).

PBMCs were grown in 48-well plates and activated with an anti-CD3 (2.0 µg/ml) ± CD28 (3.0 µg/ml) or CD3 (2.0 µg/ml) ± CD46 (2.0 µg/ml) for 24 h. Intracellular C3aR expression was measured in Treg cells and data show the mean ± SD derived from ten experiments (n=10) for each group.

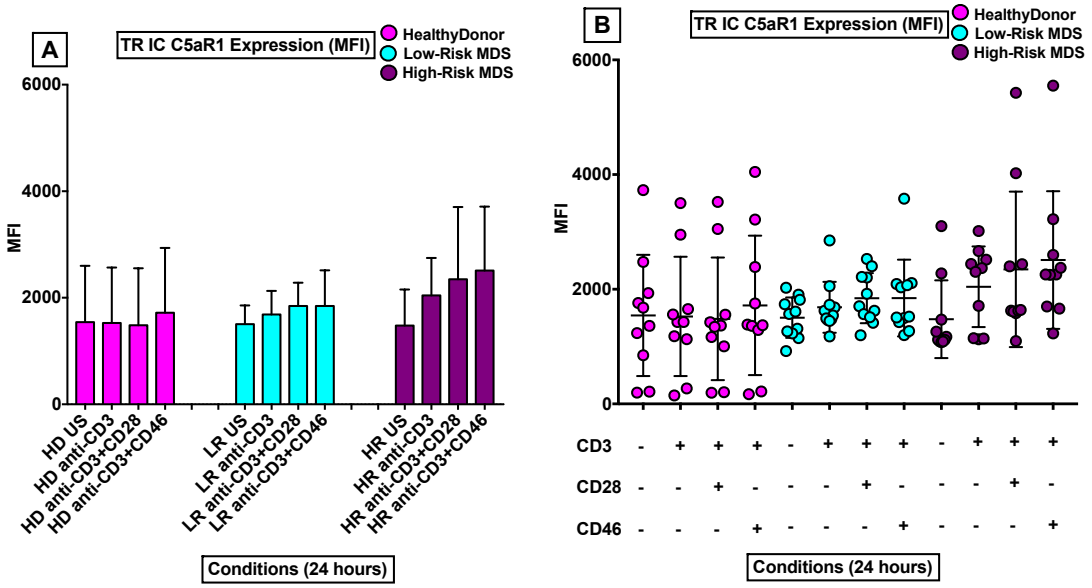


Figure 3-S3. Intracellular C5aR1 participate in human Treg cells FACS
Results show flow plots of PBMCs which were non-activated or activated for 24 h with an anti-CD3, anti-CD3±CD28 or anti-CD3±CD46 antibody. Treg intracellular C5aR1 expression was measured at indicated time point. The data show the mean ± SD derived from healthy donors (n=10), low-risk MDS (n=10) and high-risk MDS (n=10) experiments (plots are representative of a total of 30 experiments (n=30).

Intracellular CD46 and C5L2 expression in human Tconv cells

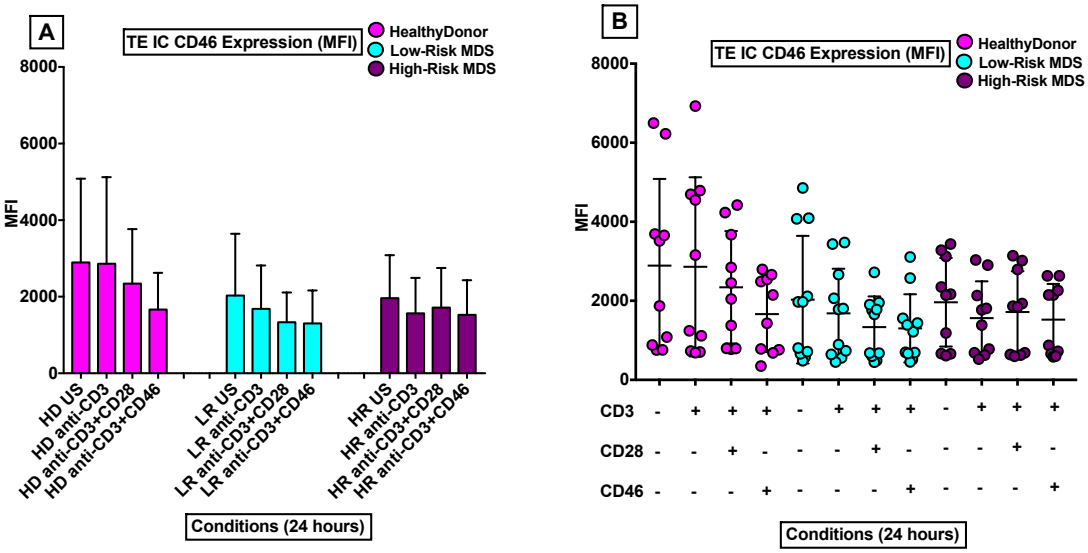


Figure 3-S4. Intracellular CD46 expression in human Tconv cells using FACS analysis.
PBMCs were cultured in 48-well plates and then stimulated with mAbs anti-CD3, anti-CD3±CD28 or anti-CD3±CD46 antibodies. Intracellular CD46 expression in Tconv cells was monitored at 24 h. The data show mean ± SD derived from ten experiments (n=10) for each group.

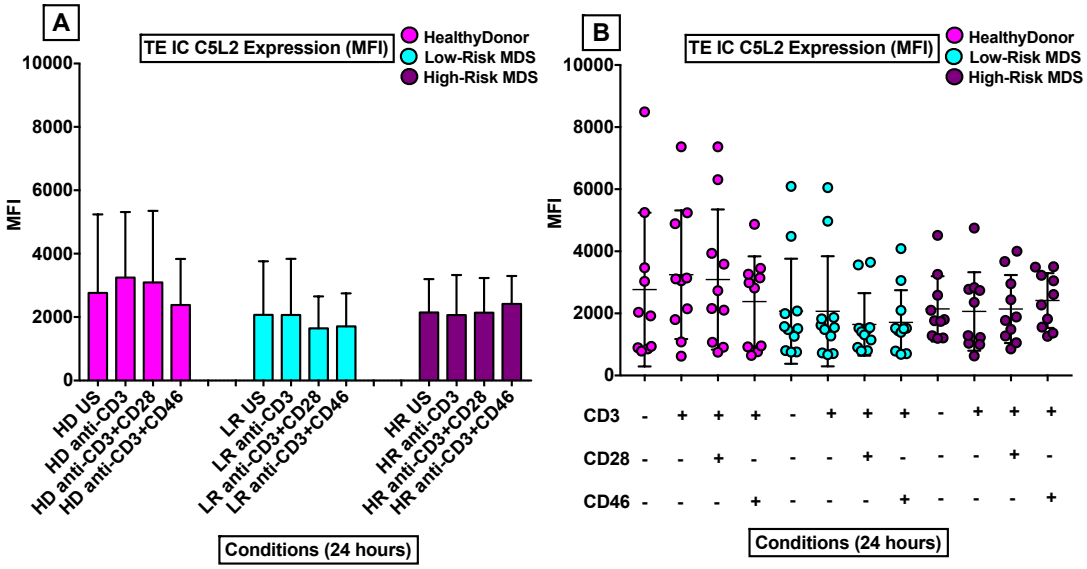


Figure 3-S5. Intracellular expression of C5L2 in human Tconv cells using FACS analysis. PBMCs were activated for 24 h with mAbs anti-CD3, anti-CD3±CD28 or anti-CD3±CD46 antibodies. Intracellular C5aR2 (C5L2) expression in healthy donor, low-risk-MDS and high-risk MDS Tconv cells was measured at different time points by FACS analysis. The data show the mean ± SD derived from healthy donor (n=10), low-risk MDS (n=10) and high-risk MDS (n=10) experiments.

Expression of CD46 and C5aR1 on the surface of Treg

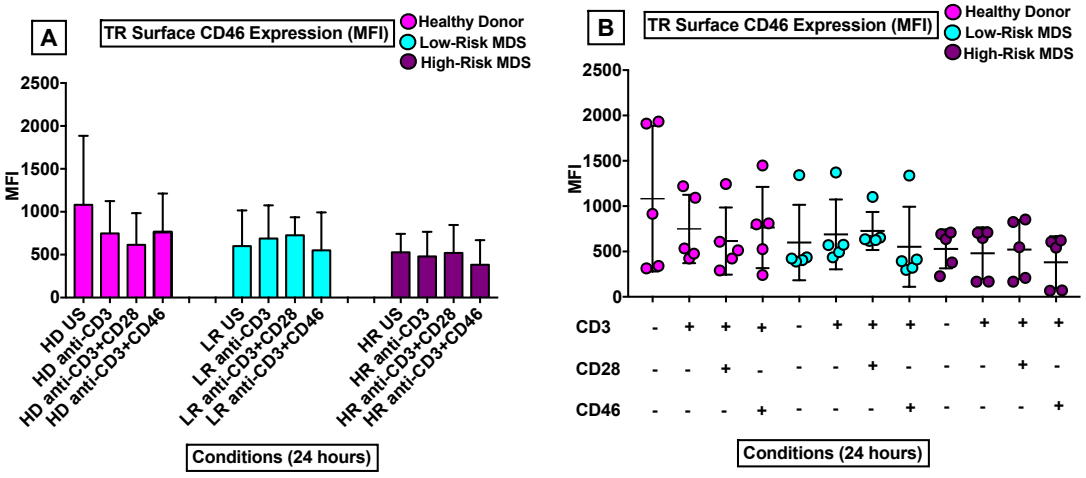


Figure 3-S6. CD46 expression on the surface of human $CD4^{+}CD25^{+}$ T-cells using flow-cytometry analysis (FACS).
PBMCs were cultured in 48-well plates and then stimulated with mAbs anti-CD3, anti-CD3±CD28 or anti-CD3±CD46 antibodies. Surface CD46 expression of T-cells (Treg) was monitored at 24 h. The data show the mean ± SD derived from five samples (n=5) for each group.

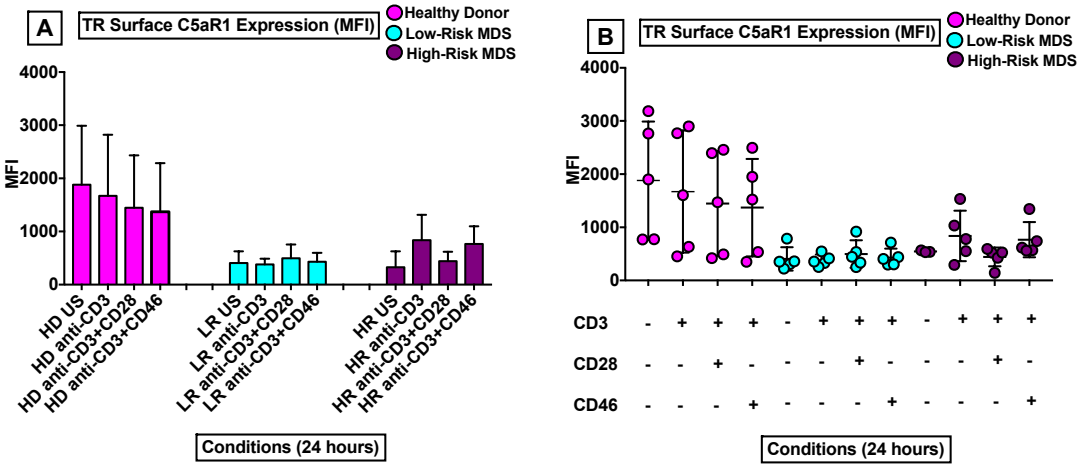


Figure 3-S7. Expression of C5aR1 on the surface of Tregs.
PBMCs were activated for 24 h with an anti-CD3±CD46 antibody. C5aR1 expression on the surface of Treg cells was measured at 24 h. The data show the mean ± SD derived from ten experiments (n=5) for each group.

Expression of C3aR on the surface of Tconv

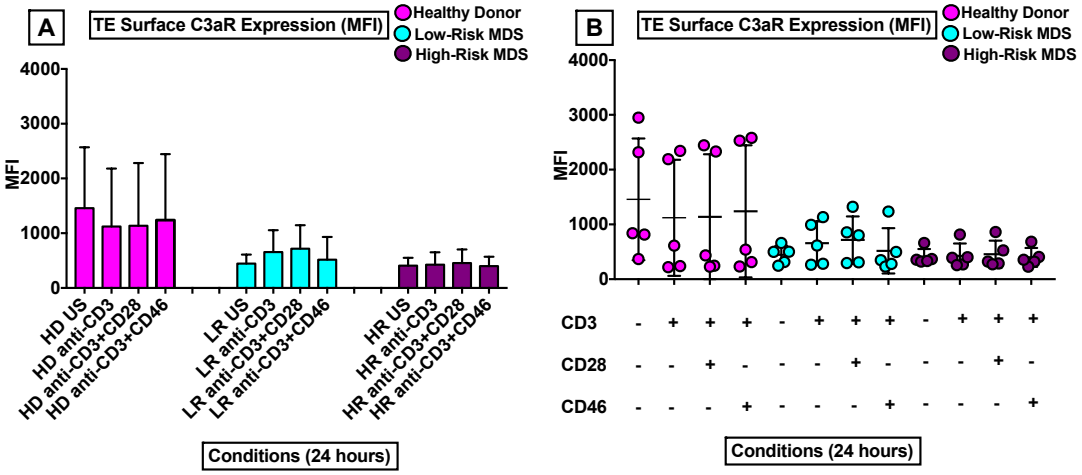


Figure 3-S8. Expression of C3aR on the surface of human Tconv cells by flow cytometry analysis (FACS).

PBMCs were grown in 48-well plates and activated with an anti-CD3 (2.0 µg/ml) ± CD28 (3.0 µg/ml) or CD3 (2.0 µg/ml) ± CD46 (2.0 µg/ml) for 24 h. C3aR expression was measured on the surface of Tconv cells and the data show the mean ± SD derived from ten experiments (n=5) for each group.

Appendix 2

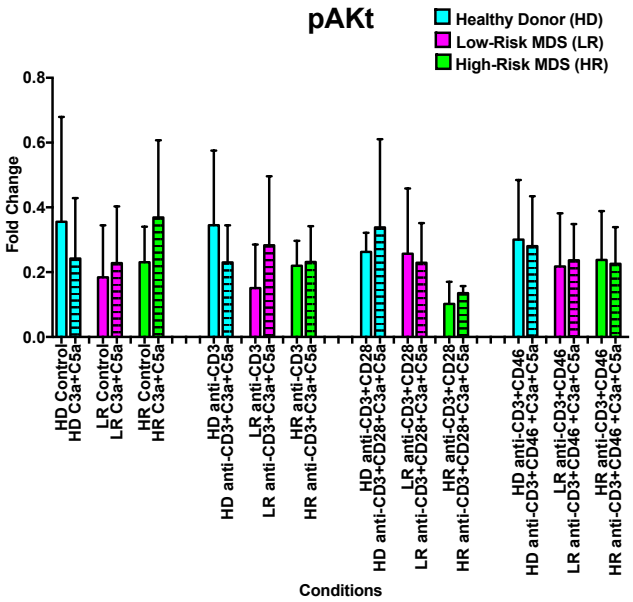


Figure 4-S1. pAKt levels expression in human naive CD4$^+$ T-cells.

Naïve CD4$^+$ T-cells from healthy donor, low-risk MDS and high-risk MDS were left inactivated or activated for 24 h with mAbs anti-CD3, anti-CD3±CD28 or anti-CD3±CD46 antibodies and cells were untreated or treated with 50nM C3a and C5a for each. The data show the mean ± SD derived from healthy donor (n=6), low-risk MDS (n=6) and high-risk MDS (n=6) experiments. Data are representative of six experiments with similar results.

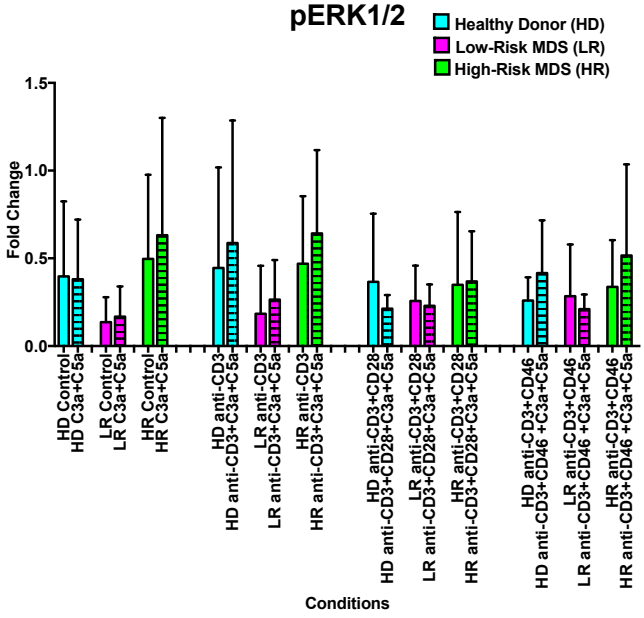


Figure 4-S2. pERK1/2 levels expression in human naive CD4$^+$ T-cells.

CD4$^+$ T-cells, from healthy (n=6), low-risk MDS (n=6) and high-risk MDS donors (n=6) without (−) or with (+) stimulation (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of 50nM C3a and C5a for each. Error bars indicate SD of six experiments. Data are representative of six experiments with similar results.

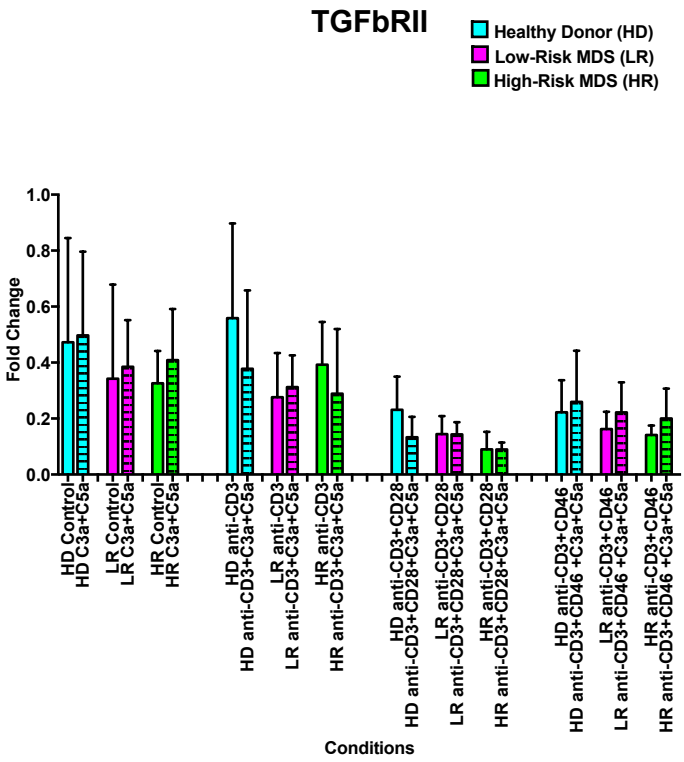


Figure 4-S3. The levels of TGFbRII

Naïve $CD4^+$ T-cells isolated from PBMCs of healthy (n=6), low-risk MDS (n=6) and high-risk MDS (n=6) donors without (−) or with (+) stimulation (anti-CD3/28) or (anti-CD3/46) and with absence (−) or with presence (+) of 50nM C3a and C5a for each. Error bars indicate SD of six experiments. Data are representative of six experiments with similar results.

International Symposium

A. European Academy of Allergy and Clinical Immunology, EAACI, Münich, Germany 26-28 May 2018

1. Oral patent poster in poster patent workshop. My name has been listed in **page 142** under Patent section **(#1634).**
2. Oral poster and my name listed on **page 123** under the Late-Breaking section **(#1742).**

B. American Society of Hematology (ASH), San Diego, California, USA, 3-6 December 2016

My abstract has been accepted in ASH 2016, Blood Journal.

Subject:	Fw: EAACI Congress 2018: Notification of Poster Patent Workshop presentation Please confirm your attendance
From:	Samia Al Shouli (samiashouli@yahoo.com)
To:	samiashouli@yahoo.com;
Date:	Monday, April 30, 2018 12:30 PM

On Monday, April 30, 2018 12:15 PM, "Alshouli, Samia" <samia.al-shouli@kcl.ac.uk> wrote:

From: "eaaci2018patent@eaaci.org" <eaaci2018patent@eaaci.org>
Date: Wednesday, 28 March 2018 at 16:50
To: Samia AL-Shouli <samia.al-shouli@kcl.ac.uk>
Subject: EAACI Congress 2018: Notification of Poster Patent Workshop presentation – Please confirm your attendance

European Academy of
Allergy and Clinical Immunology

Notification of poster patent workshop presentation

Dr. Samia Al-Shouli
United Kingdom
Dear Dr. Samia Al-Shouli,
On behalf of the Scientific Programme Committee we are pleased to inform you that your patent abstract has been accepted for presentation in a **Patent Workshop** at the EAACI Congress 2018, to be held in Munich, Germany, 26-30 May 2018.

Your patent abstract title: The Role Of Intracellular Complement In The Induction Of Ineffective CD4+ T Cell Mediated Immune Responses In Myelodysplastic Syndrome (MDS)

Your patent abstract has been scheduled in the following session:

Session details

Session type: Patent Workshop
Session title: Poster patent workshop: Innovations in Allergy
Session date: Monday, 28 May 2018
Session time: 12:15-13:45
Session room: Thematic Poster Exhibition (on exhibition floor)

Following the "EAACI Goes Green Initiative" in 2018 all posters are submitted in an electronic format and presented as E-Posters. Paper posters will NOT be accepted anymore
The patent abstracts are organised in a patent workshop session of 14 posters and will be displayed for one day at a E-Poster station. At lunch time the presenters are required to stand by their station and answer questions from delegates. During this time, two moderators will also ask the presenters to briefly present their findings. Please note that the patent workshop session runs for 90 minutes in total.
E-Posters have to be uploaded prior to the presentation in order to allow technical processing. You will be sent further instructions in April 2018. E-Posters will be also available for viewing in the EAACI Congress App as of 26 May 2018 and onsite at the congress in the Virtual Congress Hubs.

Registration
Presenting authors are required to register for the Congress and pay the registration fee. As a presenting author you are entitled

1840 **Predictors of positive challenge with kiwi in children.**
Maria Perez Sabido, University and Polytechnic Hospital La Fe, Spain

1841 **SOTI in IgE Mediated Cow's Milk Allergy, 2 case reports**
Elif Karakoc-Aydiner, Marmara University, Pendik Research and Training Hospital, Turkey

1842 **Evaluation of the quality of life in patients affected by nichel sulphate system allergy (SNAS) before and after specific desensitization**
Alessia Di Rienzo, Fondazione universitaria A. Gemelli, Italy

1843 **Fecal nitric oxide and zonulin in children with protein -induced enteropathy**
Nelli G. Prikhodchenko, The Pacific State Medical University, Russia

1844 **Zonulin, TNF-a and nitric oxide in the Ig E-dependent and Ig E-independent food allergy in children**
Nelli G. Prikhodchenko, Pacific State Medical University, Russia

1845 **Diet-induced Obesity Potentiates IgE-mediated Food Allergic Responses**
Maryam Hussain, University of Bern, Switzerland

1846 **In vitro characterization of the immune induction capacity of dietary food proteins after gastric digestion**
Nazanin Samadi, Department of Pathophysiology and Allergy Research, Medical University Vienna, Austria

1847 **Cow's milk allergy and lactose malabsorption in children with inflammatory bowel diseases**
Svetlana Makarova, FSAI, Russia

Poster patent abstract session: Innovations in Allergy

Poster Exhibition

Chairs: Ulrich Matthias Zissler, Germany
Sabine Flicker, Austria

1623 **Epigenetic Markers For Respiratory Allergy (WO 2016193151 A1)**
Sabine Langie, VITO-Sustainable Health, Belgium

1624 **Construction Of Novel Hypoallergens For Allergen Immunotherapy**
Pekka Mattila, Desentum Oy, Finland

1625 **Composition With Bacterial Lysate For Atopic Dermatitis Primary Prevention**
Pavel Nesmiyanov, Volgograd State Medical University, Russia

1626 **ALLERGY-SPECIFIC IMMUNOTHERAPY COMPOSITIONS FOR USE IN THE TREATMENT OF HOUSE-DUST MITE ALLERGY**
Adrianus Van Der Graaf, Citeq BV, The Netherlands

1627 **An Efficient Plant-Based Molecular Allergen Bearing Bioparticle Production Platform To Redefine Allergy Immunotherapy**
Louis-Philippe Vézina, Anqany Inc., Canada

1628 **Peptide Biomarkers To Predict Safety And Efficacy Of Cow's Milk Oral Immunotherapy**
Montserrat Fernandez-Rivas, Hospital Clinico San Carlos, IdISSC, ARADyAL, Spain

1629 **Controlling products contained in container devices; PCT/EP2016/056443 (monitoring epinephrine autoinjectors for anaphylaxis management)**
Anna Sala-Cunill, Allergy Section, Medicine Department, Hospital Universitari Vall d'Hebron, Spain

1630 **TABLETS FOR ORAL IMMUNOTHERAPY IN PATIENTS WITH IGE-MEDIATED COW'S MILK ALLERGY**
Stefania Arasi, University of Messina, Italy

1631 **First Hypoallergenic Propolis Extract (Hypolis): Inhibitory Effect On Most Resistant Brucella Strains And Synergism With Antimicrobial Drugs**
Maryam Dadar, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Iran

1632 **A Chimeric IgE That Mimics IgE From Patients Allergic To Acid-Hydrolyzed Wheat Proteins Is A Novel Tool For In Vitro Allergenicity Assessment Of Functionalized Glutens**
Olivier Tranquet, INRA, France

1633 **The Anaphylaxis Imaging Cage: Temperature And Activity Imaging Device For Laboratory Animals**
Erika Jensen-Jarolim, Medical University Vienna, Austria

1634 **The Role Of Intracellular Complement In The Induction Of Ineffective CD4+ T Cell Mediated Immune Responses In Myelodysplastic Syndrome (MDS)**
Dr. Samia T. Al-Shouli, King's College London (KCL) and King's College Hospital (KCH), United Kingdom

1635 **Blister-Strip Device For Simplified Skin Intracutaneous Testing A New Device For Simplified Skin PRICK Testing (SPT) Forstner B., Wels – Pneumological Practice, Alltest GmbH, Austria**
Bernhard Forstner, Pneumological practice, Wels, Austria, Austria

1636 **Adrenaline Auto-Injector**
Michael Levin, Red Cross Hospital; University of Cape Town, South Africa

Patent Abstract
EAACI 2018
26-30 Munich, Germany

The Role of Intracellular Complement in the Induction of Ineffective CD4+ T Cell Mediated Immune Responses in Myelodysplastic Syndrome (MDS)

Authors: Dr. Samia T. Al-Shouli[1*2], Dr. Shahram Y. Kordasti[1], Professor Ghulam J. Mufti[1]
[1] King's College London (KCL) and King's College Hospital (KCH), London, United Kingdom
[2] King Saud University (KSU) and King Khalid University Hospital (KUH), Riyadh, Kingdom of Saudi Arabia

Background: T cell-mediated immune dysregulation is an important feature of myelodysplastic syndrome (MDS). The expansion of regulatory T cells (Tregs) is one of the important factors in the progression of intermediate/high-risk MDS to acute myeloid leukemia. However, the exact mechanism for the expansion of Tregs in MDS is unknown. Intracellular complements (particularly C3a and C5a) play a crucial role in the polarization of CD4⁺ T cells toward regulatory or effector phenotypes through TGF-β pathway (C5aR2 mediated) or mTOR (C5aR1 mediated) respectively.

Aim: The main aim of this project was to investigate the potential role of intracellular complement system in polarisation and function of CD4 + T cells in MDS.

Result:

Table 1: Summary of the results of intracellular and surface staining of complement receptors in Tregs and Tconv cells

Complement receptor	Intracellular staining		Surface staining	
	Treg	Tconv	Tregs	Tcon
CD46	↑HD than LR & HR	→ HD, LR & HR	↑HD than LR & HR	↑↑HR than LR
C3aR C5aR1	↑HR than LR & HD	↑↑HR than LR & HD	↑↑HD than LR & HR ↑HD than LR & HR	↑HR than LR & HD ↑↑HR & HD than LR
C5L2	↑↑HR than LR & HD	→ HD, LR & HR	↑HD than LR & HR	↑↑HR & HD than LR

↑ is high; ↑↑ is significantly higher; → is similar levels; HD: healthy donors; HR: high risk MDS; LR: low risk MDS.

Figure 1: CD46 activation on high-risk MDS Treg cells induces intracellular C5L2 upregulation

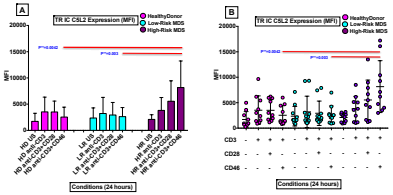


Figure 2: Western Blot analysis for Complement Components (C5a) and (C3a)

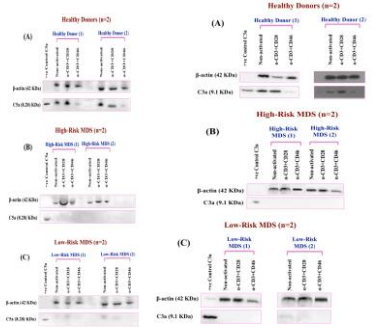


Figure 3: The role of complements components in levels of mTOR and TGF- β signalling Pathway-Associated Proteins in Patients with MDS

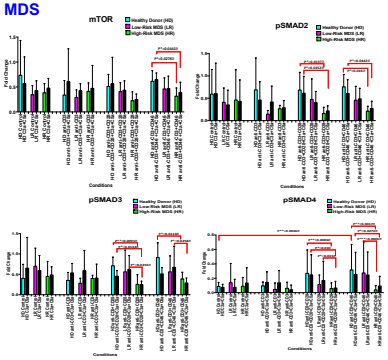
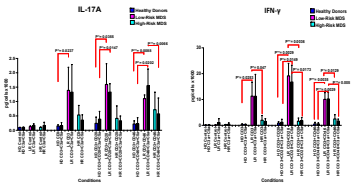


Figure 4: Cytokine secretion in response to stimulation to complement pathway stimulation



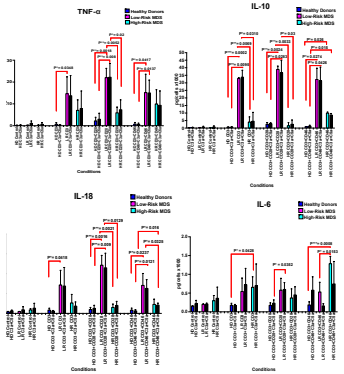


Table 2: Upregulated signalling pathways after anti-CD46 stimulation in healthy donors

Gene name	FDR q-value
Complement system	5.26×10^{-2}
PI3K/AKT signalling	5.28×10^{-2}
PAK signalling	2.18×10^{-2}
Tec Kinase signalling	4.08×10^{-2}
TNFR2 signalling	4.95×10^{-2}
HMGB1	6.61×10^{-2}
Role of pattern recognition receptors in recognition of bacteria and viruses	6.45×10^{-2}
Notch signalling	6.92×10^{-2}
Molecular mechanisms of cancer	6.04×10^{-2}
Mitochondrial dysfunction	3.23×10^{-2}

Figure 5: Significant genes functions of Tconv cells in HD

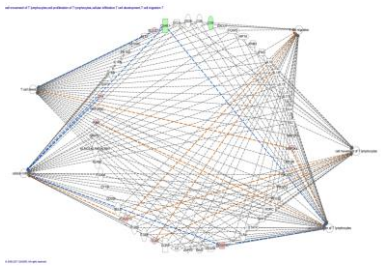


Figure 6: Rheumatoid arthritis pathway in MDS patients

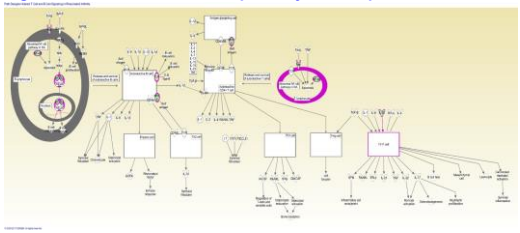


Figure 7: TREM1 pathway in MDS patients' vs healthy donors after anti-CD3+CD46 stimulation. The green is inhibition and the red is increased expression.

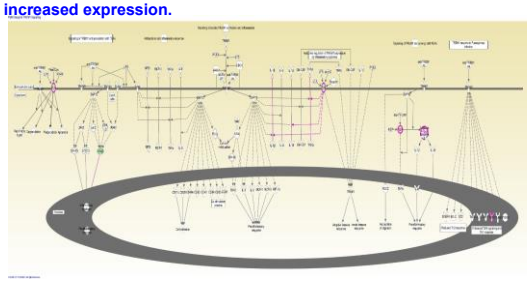
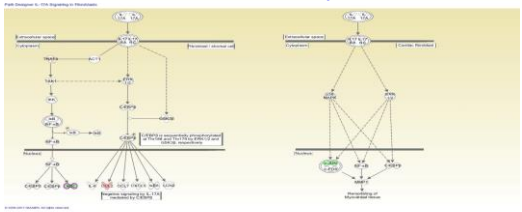


Figure 8: Signaling pathway in MDS patients' vs healthy donors after anti-CD3+CD46 stimulation with complement.



Conclusion:

Altogether, this work shows for the first time the potential role of complement in CD4 + T cells polarisation in MDS patients and provides data on two major pathways that may be implicated in the lack of complement in T cells of MDS patients. Future work to explore these further may help to identify potential molecules involved which can serve as potential therapeutic targets in MDS in the future.

0943 **Late reaction to specific immunotherapy? Not all is allergy.**
Virginia De Luque, Virgen Macarena Hospital, Spain

0944 **Heterogeneity of commercial house dust mites extracts**
Giuseppe Di Cara, Università degli Studi di Perugia - Dipartimento di Scienze Chirurgiche e Biomediche, Italy

0945 **Treatment effect of grass-pollen immunotherapy for children with allergic rhinoconjunctivitis in a district general hospital**
Paul Watson, Worcestershire Acute Hospitals NHS Trust, United Kingdom

0946 **Clinical analysis of specific immunotherapy for allergic asthma**
Xiaoyang Liu, China-Japan Union Hospital of Jilin University, China

0947 **Application of component resolved diagnostics in treatment of severe atopic dermatitis**
Delara Babaie, Department of Allergy and Clinical Immunology, Mofid Children's Hospital, ShahidBeheshti University of Medical Sciences, Tehran, Iran., Iran

0948 **House dust mite immunotherapy and remission of chronic urticaria: Better if a conjuncture?**
Doina Piriu, Medstar General Hospital Constanta,Allergy Department, Romania

0949 **Efficacy of cat sublingual immunotherapy in a Thai girl with severe asthma**
Piernpit Likkasittipan, Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand

0950 **Candida immunotherapy in a patient with severe atopic dermatitis**
Payam Payandeh, Allergy research center. Mashhad university of medical science, Iran

0951 **Rheumatoid arthritis developed during subcutaneous pollen allergen immunotherapy; a case report**
Ayse Fusun Kalpaklioglu, Kirikkale Universty Faculty of Medicine, Division of Immunology and Allergy, Turkey

0952 **The importance of molecular diagnosis in allergen-specific immunotherapy.**
Pilar Lara De La Rosa, Clinical Management Unit of Allergology. University Hospital Virgen Macarena., Spain

0953 **Adaptive immune responses in allergic subjects in response to natural allergen exposure**
Julia Eckl-Dorna, Medical University of Vienna, Austria

Late Breaking Thematic Poster Session (LB TPS 01) – Immunity and regulators of health and disease

Poster Exhibition

Chairs: Hermelijn Smits, The Netherlands
Lawrence DuBuske, United States

1735 **Epinephrine-driven upregulation of ß2-adrenergic receptor shifts human M2a allergic macrophages toward a regulatory phenotype**
Christina Pranger, Comparative Medicine, The Interuniversity Messerli Research Institute of the University of Veterinary Medicine Vienna, Medical University Vienna and University Vienna; Institute of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Austria

1736 **Skin stem cells express GATA3 independent of the IL-4 receptor expression**
Ileana Ghiordanescu, Center of Allergy and Environment (ZAUM), Technical University and Helmholtz Center, Germany

1737 **The effect of high dose vitamin-D on lymphocytes in children with atopic dermatitis**
Elif Karakoc-Aydiner, Marmara University, Turkey

1738 **Could immunoglobulins be markers of pediatric SLE activity?**
GEANINA IRINA Criscov, UMF, Romania

1739 **Genetic variants and molecular biomarkers related with ateromatosis in pediatric lupus nephritis (pLN) - in search of risk factors.**
Eduardo Egea, Department of Medicine; Universidad del Norte. Faculty of Health Sciences; Universidad Simón Bolivar, Colombia

1740 **Glycosylation pattern of IgE and its potential impact on different diseases**
Linlin Yang, Center for Chronic Immunodeficiency (CCI), University Medical Center Freiburg, Germany

1741 **The efficacy of the combine program of the interferon- and immunomodulatory therapy in patients suffering from atopic asthma and recurrent acute viral respiratory infections**
Irina Nesterova, Peoples' Friendship University of Russia, Russia

1742 **Comparative study of Complement Components, Regulators and Receptors in Healthy Donors vs. MDS Patients Authors: Dr. Samia T. Al-Shouli, Dr. Shahram Y. Kordasti, Professor Ghulam J. Mufti**
Dr. Samia T. Al-Shouli, King's College London (KCL) and King's College Hospital (KCH), United Kingdom

1743 **Correlation of hepatitis B and C virus infection with pancreatic tumor markers**
Nino G Gachechiladze, Iv. javakhishvili Tbilisi State University, Georgia

1744 **Energy consumption versus cytokine concentrations in elite athletes**
R Khanferyan, Peoples' Friendship University of Russia, Russia

1745 **Visualizing the effect of different osteopathic pump techniques on t helper lymphocytes in normal subjects "randomized control trial"**
Ahmed Abdelfattah, faculty of physical therapy - cairo university, Egypt

1746 **Evaluation of miR-340 expression in Bt474 Trastuzumab resistant cell**
Zohreh Rezaei, Department of Biology, University of Sistan and Baluchestan, Iran

1747 **Human rhinovirus coinfection with Mycoplasma pneumoniae in children with pneumonia**
Hao Chuangli, Children's hospital of Soochow University, China

Comparative study of Complement Components, Regulators and Receptors in Healthy Donors vs. MDS Patients

Authors: Dr. Samia T. Al-Shouli[1*2], Dr. Shahram Y. Kordasti[1], Professor Ghulam J. Mufti[1]
[1] King's College London (KCL) and King's College Hospital (KCH), London, United Kingdom
[2] King Saud University (KSU) and King Khalid University Hospital (KUH), Riyadh, Kingdom of Saudi Arabia

Background: Myelodysplastic syndrome (MDS) is a relatively uncommon clonal haematological disease that is characterised by peripheral blood cytopenias despite a normal or hypercellular bone marrow. The disease which is common in the elderly transforms to acute myeloid leukaemia in up to 40% of cases. Several studies have established that the T-cell mediated immune dysregulation is an important feature of MDS. Low risk MDS is associated with a proinflammatory environment and an increase in IL-17 producing T-cells as well as an increase in the serum IL-17, IL-12, RANTES and IFN-γ. In contrast, IL-10 (inhibitory factor) and IL-2R (soluble receptor) are increased in high risk MDS. A high number of IL-17 producing CD4+ T-cells are found in low risk MDS compared to the high-risk disease where expansion of T regulatory cells (Tregs) is the main feature. However, the mechanism for this switch in the immune signature is not fully understood. Immune cell derived complement activation fragments are recently identified as key players in driving and modulating adaptive immunity, C3a and C3b. C3 fragments C3a and C5a are particularly important in induction of IFN-γ secretion through autocrine engagement of the C3a receptor and CD46 (C3b receptor). The activation pathway following CD46 activation varies between Tconv and Tregs and the balance between these pathways is crucial for the Treg/Tconv equilibrium. The objective of this study was to elucidate the role of intracellular complement components (C3a and C5a), regulators (CD46) and their receptors (C3aR, C5aR1 and C5aR2) in the expansion of T regulatory cells, which is one of the main factors in the progression HR MDS patients towards AML.

Method: PBMCs and $CD4^+$ T-cells from healthy controls (n=10), low-risk (n=10) and high-risk MDS patients (n=10) were used for this study. Anti-CD3 (2.0 µg/mL), anti-CD28 (3.0 µg/mL) and/or anti-CD46 (2.0 µg/mL) antibodies were used to stimulate cells or were deliberately left non-activated. Flow cytometry was used to monitor the intracellular expression of C3aR, C5aR1, C5aR2 (C5L2) and CD46 (C3b receptor), as well as their surface expression on T-conventional and Treg $CD4^+$ T-cells. Western blot was used to evaluate C3a and C5a expression of cultured $CD4^+$ T-cells.

Result: Tregs were stimulated with anti-CD3+CD46 expressed a significantly higher level of intracellular C5aR2 in high-risk MDS compared to low-risk MDS (P=0.003) and healthy donors (P=0.0042). Thus, CD46 induced an upregulated C5aR2 expression of Tregs in high-risk MDS patients. Western blotting demonstrated that in healthy donors the stimulation of $CD4^+$ T cells with anti-CD3+CD46 resulted in downregulation of both C5a and C3a compared to non-activated or anti-CD3+CD28 stimulation. In both high and low-risk MDS donors, there was no expression of these two complement components whether the cells were activated or non-activated.

Table 1. Summary of the results of intracellular and surface staining of complement receptors in Tregs and Tconv cells

Complement receptor	Intracellular staining		Surface staining	
	Treg	Tconv	Tregs	Tcon
CD46	↑HD than LR & HR	→ HD, LR & HR	↑HD than LR & HR	↑↑HR than LR
C3aR	↑HR than LR & HD	↑↑HR than LR & HD	↑↑HD than LR & HR	↑HR than LR & HD
C5aR1			↑HD than LR & HR	↑↑HR & HD than LR
C5L2	↑↑HR than LR & HD	→ HD, LR & HR	↑HD than LR & HR	↑↑HR & HD than LR

↑ is high; ↑↑ is significantly higher; → is similar levels; HD: healthy donors; HR: high risk MDS; LR: low risk MDS.

Figure 1. CD46 activation on high-risk MDS Treg cells induces intracellular C5L2 upregulation

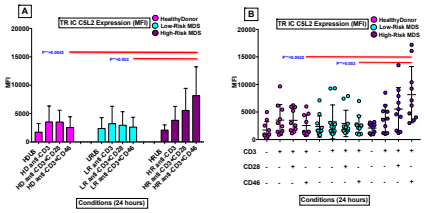


Figure 2. CD46 activation of Tconv in high-risk MDS upregulates the intracellular C3aR expression

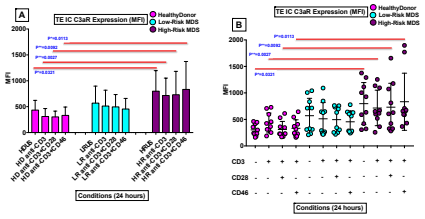


Figure 3. Post-CD46 activation of Tconv in high-risk MDS induces overexpression of intracellular C5aR1

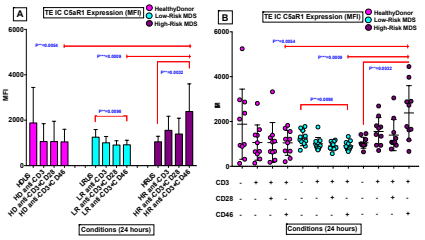
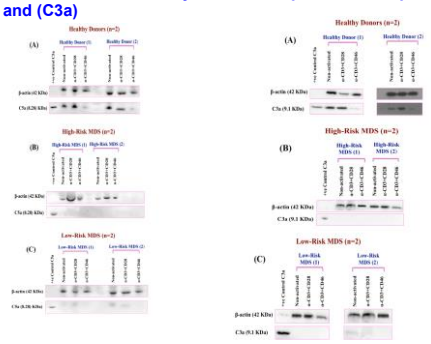


Figure 4. Western Blot analysis for Complement Components (C5a) and (C3a)



Conclusion: In this study, we observed no expression of C3a and C5a, subsequent lack of signalling through C3a and the C5a receptors, along with up-regulation of C5aR2 (an alternative receptor for C5a) in patients suggesting a potential mechanism for Treg expansion in MDS. Collectively, the results demonstrate that the insufficiency or excess of function and/or distribution of complement expression may partly contribute to inappropriate $CD4^+$ T-cells activity in MDS and these findings may lead to identification of new therapeutic targets.

European Academy of
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Reduced Levels of mTOR and TGF- β Signaling Pathway-Associated Proteins in Patients with High Risk MDSsamia AL-Shouli. , Pilar Perezabellan, Frederic Toulza. Shahram Kordasti, Ghulam Mufti

Samia Towfeek Al-Shouli and Ghulam J Mufti

Blood 2016 128:5501;

Abstract

Background:

T cell mediated immune dysregulation is an important feature of MDS. The expansion of regulatory T cells (Tregs) is one of the important factors in the progression of intermediate/high risk myelodysplastic syndrome (MDS) to acute myeloid leukemia. However, the exact mechanism for the expansion of Tregs in MDS is not known. Intracellular complements (particularly C3a and C5a) play a crucial role in the polarization of CD4+ T cells toward regulatory or effector phenotypes through Transforming growth factor-beta (TGF-β) pathway (C5aR2 mediated) or Mammalian Target of Rapamycin (mTOR)(C5aR mediated) respectively. The aim of this study was to investigate the potential role of mTOR and Akt as important proteins in complement related polarization of CD4+ T cells toward pro-inflammatory T helper cells in MDS. We have also studied the TGF-β signaling pathway related proteins, which are crucial for the expansion of Tregs. We investigated the level of TGF- β related proteins (phosphorylated (p) SMADs), as well as mTORc and Akt (Ser473) in high risk MDS and healthy donors (HD) before and after stimulation with CD3 and CD46 as a

complement related co-stimulatory molecule.

Methods:

Peripheral blood mononuclear cell (PBMCs) from healthy controls and high-risk MDS patients were used for this study. Anti-CD3 (2.0 µg/mL), anti-CD28 (3.0 µg/mL) and/or anti-CD46 (2.0 µg/mL) antibodies were used to stimulate cells. The total protein was extracted by Bicinchoninic Acid (BCA) assay and quantified by nano-drop. The MILLIPLEX MAP Human TGF-β Signaling Magnetic Bead Panel 6-plex was used to detect the signaling changes in cell lysates using the Luminex® system following the manufacturer's instructions. Data were analysed using Microsoft Excel and expressed in means and standards deviation. The students T-test were used to assess the difference in means between groups.

Results:

TGF-β signaling pathway proteins pSMAD2, pSMAD3 and pSMAD4 as well as mTORc were evaluated. Unstimulated PBMCs from high-risk MDS patients showed a significantly lower level of m-TOR ($p=0.01$), pSMAD2 ($p=0.01$), pSMAD3 ($p=0.02$) and pSMAD4 ($p=0.044$) as compared to healthy donors. Following stimulation with anti-CD3±CD46 for 24 hours, there was no significant increase in protein levels of mTORc or Akt. However, in high-risk MDS patients the level of pSMAD2 ($p=0.02$) and pSMAD4 ($p=0.006$) remain significantly lower than healthy donors after 24 hours of stimulation with anti-CD3 and CD46.

An aliquot of cells were used for flowcytometry following stimulation. Interestingly Tregs phenotype CD4+CD25^{high}CD127^{low}expressed higher level of intracellular C5aR2 in MDS ($n=5$) compared to HD ($n=5$).

Conclusion:

mTORc protein level in MDS is reduced and does not change in response to complement receptor stimulation neither does the level of Akt. This may prevent T cells to polarize toward pro-inflammatory T cells (Th1 or Th17) therefore avert an effective immune-surveillance against malignant clone. Lack of response to complement related co-stimulation and increase in C5aR2 expression suggest a potential mechanism for Treg expansion in MDS. These findings may lead to identification of new therapeutic targets in MDS, although need further studies on larger cohort of patients.

Disclosures No relevant conflicts of interest to declare.

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Clonal Development of Hematopoietic Stem Cell Specification and Differentiation at Single Cell Resolution	2
Special Symposium on the Basic Science of Hemostasis and Thrombosis	2
AML According to WHO: Newer Approaches to Therapy	1.5
AML According to WHO: Newer Approaches to Therapy	1.5
Pediatric Hematologic Malignancies	1.5
Optimizing Treatments for High-Risk Acute Lymphoblastic Leukemia: What It Takes to Move the Needle	1.5
Optimizing Treatments for High-Risk Acute Lymphoblastic Leukemia: What It Takes to Move the Needle	1.5
Myelodysplastic Syndromes	1.5
Myeloproliferative Neoplasms	1.5
Precision Medicine Trials as a Novel Approach to Drug Development	1.5
Complement Disorders and Hematologic Consequences	1.5
Complement Disorders and Hematologic Consequences	1.5
Presidential Symposium	1.5
Ham-Wasserman Lecture	1
ASH-EHA Joint Symposium	1
Ernest Beutler Lecture and Prize	1
E. Donnall Thomas Lecture and Prize	1
Featured Topic: Novel Approaches to Immunotherapy Not Just Checkpoint	1
Disorders of Complement	1
Pathogenesis of Myeloproliferative Disorders	1
Plenary Scientific Session	2
Late-Breaking Abstracts Session	1.5
102. Regulation of Iron Metabolism: Hepcidin and the Regulation of Iron Homeostasis	1.75
203. Lymphocytes, Lymphocyte Activation, and Immunodeficiency, including HIV and Other Infections: B-Cell Homeostasis and T-Cell Signaling	1.5
602. Disordered Gene Expression in Hematologic Malignancy, including Disordered Epigenetic Regulation: DNA-Methylation	1.5
603. Oncogenes and Tumor Suppressors: Novel Signaling Pathways in Hematological Malignancies	1.5
721. Clinical Allogeneic Transplantation: Conditioning Regimens, Engraftment, and Acute Transplant Toxicities: Conditioning Regimens	1.75
722. Clinical Allogeneic Transplantation: Acute and Chronic GVHD,	1.5

Immune Reconstitution: Phase I Trials	
Poster Session I - Presentations	2
Poster Session II - Presentations	2
Poster Session III - Presentations	2

Total CME Credit Hours Evaluated: 52
Total CME Credit Hours Earned (maximum): 37.5

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